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BIOLOGICAL HYDROGEN PRODUCTION USING ORGANIC WASTE AND SPECIFIC BACTERIAL SPECIES

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ABSTRACT

Hydrogen (H_2) is a valuable gas required as feedstock for several industries that can also be used as a clean energy source. Demand on hydrogen production has increased considerably in recent years. Biological hydrogen production (BHP) appears to be very promising as it is non-polluting process and hydrogen can be produced from water and biodegradable wastes.

Dark fermentation is the biological process for hydrogen production showing the highest potentials for sustainable hydrogen production, because of its high production rates in the absence of a light source and thank to the possibility of using a variety of different organic substrates. Furthermore, the efficiency of energy production can be improved by screening microbial diversity and easily fermentable feed materials.

In this sense, the present study reports a research on biological hydrogen production from a real mixed bacteria culture, represented by three specific types of granular sludge, and from pure cultures constituted of five dark fermentative bacteria (*Bacillus licheniformis*, *Paenibacillus cookie*, *Bacillus* sp., *Paenibacillus* sp. and *Bacillus farraginis*). These hydrogen production tests were performed in batch reactors under mesophilic conditions.

The highest hydrogen production was measured by the mixed bacteria culture, represented by the sample "Sludge 2013", with a total hydrogen production of 148.2 NmlH_2/gVS. Among the five different fermentative bacteria, *Bacillus farraginis* showed great performance with a total hydrogen production of 95.2 NmlH_2 /gVS.

Furthermore, other results showed that the efficiency of the hydrogen production was decreased by increasing glucose concentration. It was also proven that the most efficient condition to obtain the higher hydrogen production is the addition of Nutrient Broth (NB), in a first run, in order to provide the necessary nutrients for the bacteria, and subsequently add glucose as carbon source.

Future researches may be interesting, in order to obtain an inoculum that is a consortium of bacteria characterized by high potentials for hydrogen production for further scale-up and industrial application.

Keywords: Hydrogen; Dark fermentation; Mixed cultures; Pure cultures; *Bacillus.*

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ABBREVIATIONS AND ACRONYMS

BHP	Biological Hydrogen Production
BMP	Biological Methane Potential
DNS	Dinitrosalicylic
ERASMUS	European Region Action Scheme for the Mobility of University Students
FID	Flame Ionization Detector
HPM	Hydrogen Production Medium
NB	Nutrient Broth
NI	Non Inoculated
OD	Optical Density
TCD	Thermal Condutivity Detector
TKN	Total Kjeldahl Nitrogen
TOC	Total Organic Carbon
TS	Total Solids
UASB	Upflow Anaerobic Sludge Blanket
VFA	Volatile Fatty Acids
VS	Volatile Solids

PREFACE

Since I decided to enroll in the Porto University, one of my main goals was to know if I would be able to study in a different country and in a completely different environment than the one I am used to. The best moment happened in the last year of the Master in Environmental Engineering, at the completion of the dissertation.

In this sense, the ERASMUS programme was the opportunity for which I was expecting. This programme provided me an extremely enriching academic and personal experiment. Facing new reality encompasses challenges that tend to be overcome with our larger involvement in a different society. These are constant challenges which promote this international experiment. The effort to achieve the academic and personal goals makes it unique and thus, it is possible to develop single qualities such as autonomy, responsibility and curiosity for the "*know more*".

As final stage of the course, I would like to work on the topic about solid waste treatment, because it is a subject that always captured my interest and nowadays it is a field with numerous researches, which will lead to the possibility of improving the planet future, for example, in the field of sustainable energy production. Furthermore, I have always had the idea to perform my thesis in an experimental research or apply my knowledge on a work in the field.

For all these reasons, the ERASMUS programme complemented my academic path to enable the realization of my thesis at the Department of Civil, Environmental and Architectural Engineering of the University of Padova, in Italy. With the cooperation of Prof. Raffaello Cossu and Dr. Luca Alibardi, I had the opportunity to conduct my thesis in the Laboratory of Environmental and Sanitary Engineering of the University of Padova that is located in Voltabarozzo, a quartier in the south part of Padova. Dr. Luca Alibardi, my co-supervisor, proposed to me to work on biological hydrogen production (BHP) tests and so, the theme of my thesis is the *Biological Hydrogen Production using organic waste and specific bacterial species*.

In March of 2013 my research began with the following main objectives: evaluate the biological hydrogen production potentials from a real mixed bacteria culture, using granular sludge samples collected at different times from a real scale plant; evaluate the biological hydrogen production potentials from pure cultures of single bacteria species;

comparison between a mixed and a pure culture of bacteria, in terms their maximum hydrogen productions and rates; evaluate the behavior of the best mixed culture in different food/microorganisms ratio conditions.

With all the work that I performed in the laboratory I acquired skills on the lab scale procedure for the evaluation of biological hydrogen production potentials by fermentation processes and I also worked in the biological methane potential (BMP) tests, in order to understand the behavior that each sample had at the second stage of the anaerobic digestion process. This last subject will not be analyzed in this work, since the main objective is the biological hydrogen production (BHP), but the results of BMP tests can be consulted in Annex 1. Moreover I acquired competences and autonomy on physical and chemical analysis of organic materials, such as: Total Solids (TS), Volatile Solids (VS), Total Organic Carbon (TOC), Total Kjeldahl Nitrogen (TKN), Ammonium nitrogen and Total Phosphorous, Chemical Oxygen Demand (COD), Optical Density (OD), Titration at 7 points, Volatile Fatty Acids (VFA), water displacement method and qualitative biogas measurements by gascromatographic techniques.

Before starting the experimentation on the two stage anaerobic digestion process, it was necessary to decide the working conditions: the F/M ratio, the concentrations of substrate and inoculum to use, and the pH at which the tests would have been conducted. With this purpose I started my literature research on the web and on scientific articles, but I also read the previous theses done in the laboratory by other students, to understand what I could do to improve the experiment or to avoid mistakes already done. Actually the parameters were changing in every experiment, because of the great variability that substrates were displacing, or because of the different systems that were used. Sometimes it was also difficult to find a comparison between different works.

Despite all the difficulties that appeared, such as finding a house to live in, move by bicycle with rain and wind, a different language to learn and mainly be far away from my family and friends, this experiment abroad helped me not only to expand my knowledge on a theme that is nowadays as concrete as unknown, also to face the lab approach and to deal with a real research and most important to realize that I am able to overcome the most difficult challenges in my life.

1. INTRODUCTION

Hydrogen is a valuable gas that is currently used as feedstock for in several industries processes. Hydrogen is also considered to be a clean energy source. Therefore, demand on hydrogen production has increased considerably in recent years. Electrolysis of water, steam reforming of hydrocarbons and auto-thermal processes are well-known methods for hydrogen gas production, but not environmentally sustainable due to high energy requirements, mainly based on fossil fuels. In this sense, biological production of hydrogen gas has significant advantages over chemical methods (Kapdan & Kargi, 2006). In fact, this is an exciting scientific area since it is dealing with the conversion of low costs residues or organic waste to a valuable energetic source, hydrogen (Hu *et al*., 2013).

Biological hydrogen production from renewable sources has received considerable attention in recent years. The biological processes utilized for hydrogen gas production mainly are bio-photolysis of water by algae or cyanobacteria, dark fermentation and photo fermentation of organic materials. The process of hydrogen production by fermentative bacteria, not light dependent, is known as dark fermentation and takes place during the fermentative or acidogenic phase of anaerobic digestion. Dark and photo fermentation processes are considered more environmental beneficial and feasible due to simultaneous waste treatment and hydrogen production. However, dark fermentation is faster than photo fermentation and it has several advantages like no light dependency, high production rates and efficiency, it can use various organic waste and wastewater enriched with carbohydrates (Hu *et al*., 2013). On the other hand, dark fermentation represents not only an energy production process but also a first stage of stabilization for organic substrates since it degrades complex organic matter to readily biodegradable compounds (volatile fatty acids and alcohols) suitable for methane production by anaerobic digestion (Kapdan & Kargi, 2006).

Anaerobic treatment of complex organic materials is normally considered to be a two-stage process. In the first stage, the complex organics are changed in form by a group of facultative and anaerobic bacteria commonly termed the “acid formers”. Complex materials such as fats, proteins and carbohydrates are hydrolyzed, fermented and biologically converted to simple organic materials. For the most part, the end products of this first-stage conversion are organic fatty acids (McCarty, 1964).

Although no waste stabilization occurs during the first stage of treatment, it is required to place the organic matter in a form suitable for the second stage of treatment. It is in the second stage of methane fermentation that real waste stabilization occurs. During this stage, the organic acids are converted by a special group of bacteria termed the "methane formers" into the gaseous end products, carbon dioxide and methane. The largest percentage of methane will still result from acetic acid fermentation, which is the most prevalent volatile acid produced by fermentation of carbohydrates, proteins and fats. Acetic and propionic acid, on the other hand, are formed mainly during fermentation of carbohydrates and proteins. The other volatile acids, although significant are of minor importance (McCarty, 1964).

The purpose of biological hydrogen studies is to develop commercially practical hydrogen production processes by exploiting hydrogen producing ability of microorganisms through modern biotechnology (Debabrata & Nejat, 2001). Due to the fact that solar radiation is not a requirement, hydrogen production by dark fermentation does not demand much land and is not affected by the weather condition. Hence, the feasibility of the technology yields a growing commercial value. Biological dark fermentation is also a promising hydrogen production method for commercial use in the future. With further development of these technologies, biomass will play an important role in the development of sustainable hydrogen economy (Ni *et al*., 2006).

In this perspective, this study was performed, via dark fermentation, with the following main objectives: i) evaluate the biological hydrogen production potentials from a real mixed bacteria culture, using granular sludge samples collected at different times from a real scale plant; ii) evaluate the biological hydrogen production potentials from pure cultures of single bacteria species; iii) comparison between a mixed and a pure culture of bacteria, in terms their maximum hydrogen productions and rates; iv) evaluate the behavior of the best mixed culture in different food/microorganisms ratio conditions.

To maximize hydrogen production via dark fermentation, methanogens and hydrogen-consuming bacteria should be inhibited. Moreover, optimal process conditions, as type and pre-treatment of inoculum, pH, temperature and substrate characteristics, should be defined in order to promote the metabolic pathways resulting in hydrogen production (Fang & Liu, 2002).

Anaerobic sludges collected from full-scale digesters are frequently reported to be used as inocula for hydrogen production and their pre-treatments are essential for the inhibition of methanogenic microbial species (Van Ginkel & Sung, 2001; Ting *et al*., 2004; Tommasi *et al*., 2008). Several methods have been proposed to achieve this aim, including the heat treatment that is widely applied due to its effectiveness on methanogenic inhibition (Alibardi *et al*., 2009).

Therefore, in this study, the seed material used for the evaluation of the biological hydrogen production from a real mixed bacteria culture was a granular sludge collected from a real scale Upflow Anaerobic Sludge Blanket (UASB) anaerobic digester of a brewery factory located in Padova, Italy. This granular sludge used was pre-treated, based on a heating procedure, prior to starting the experimental tests as inocula of batch test for hydrogen production.

For organic materials to be potentially useful as substrates for sustainable biohydrogen production, they must be not only abundant and readily available but, also, cheap and highly biodegradable (Guo *et al*., 2010). Glucose and sucrose are the fermentation substrates most studied in the laboratory. Thus, glucose was the substrate used as carbon source, since it is a common and abundant substrate that could come from hydrolysis of starch or cellulosic feedstock. Glucose is also shown to be a very effective substrate for fermentative hydrogen production, leading to excellent hydrogen productivities.

Some species of indigenous microbial population of organic waste may have good characteristics for the hydrolysis of complex substrates into simple monomers and for an efficient conversion into hydrogen. In this perspective, organic waste could serve not only as a substrate for hydrogen production but also as a source of hydrogen producing bacteria (Favaro *et al*., 2013). Therefore, the efficiency of energy production can be improved by screening microbial diversity and easily fermentable feed materials (Kalia & Purohit, 2008).

Among the dark fermentative hydrogen producers pure cultures known to produce hydrogen from carbohydrates include species of *Enterobacter*, *Bacillus* and *Clostridium*. The latter two groups are characterized by the formation of spores in response to unfavorable environmental conditions such as lack of nutrients or rising temperature (Hawkes *et al*., 2002).

In 2011 an investigation was conducted at the Laboratory of Environmental and Sanitary Engineering of the University of Padova which aimed at the development of an efficient microbial inoculum for the industrial conversion of organic residues into hydrogen. The presence of different extracellular enzymatic activities in many *Bacillus* sp. should be considered promising towards the definition of a proper inoculum for the conversion of complex organic wastes into hydrogen (Favaro *et al*., 2011). *Bacillus* genus shows many features appropriate for hydrogen production: they can survive under harsh conditions, hence could compete with other microbes; they have large and versatile enzymatic activities such as lipase, amylase, protease and cellulose, hence a diverse range of bio-wastes could be used as substrate for bio-hydrogen production; they do not require light for hydrogen production; *Bacillus* spores are being used as probiotics in humans and animals, thus they may not pose environmental concerns (Kalia & Purohit, 2008).

In literature, indeed, *Bacillus* sp. is considered as a strong candidate for biological H_2 – production, because of its unique traits. For this purpose, *Bacillus licheniformis*, *Paenibacillus cookie*, *Bacillus* sp., *Paenibacillus* sp. and *Bacillus farraginis* were investigated and compared at different glucose concentrations. In addition, relationship between different substrates used, which were glucose and Nutrient Broth (NB), and cumulative hydrogen productions were also evaluated.

Although the microbiology and biochemistry of the anaerobic process is complex, it normally operates quite well with a minimum control. The bacteria responsible for this treatment are widespread in nature and grow well by themselves when provided with a proper environment (McCarty, 1964).

The results from this study were expected to be helpful for understanding the behavior of anaerobic hydrogen production process, using pure and mixed anaerobic bacteria cultures in batch mode.

2. EXPERIMENTAL PROCEDURES

2.1 Research Scheme

The present research work was divided into three fundamental parts related respectively with the proposed objectives, which are concretely:

Part 1 – Biological hydrogen production from a real mixed bacteria culture;

Part 2 – Experiments in a batch mixed reactor.

Part 3 – Biological hydrogen production potentials from pure cultures of single bacteria species;

All the experiments were carried out at batch level and the results were assessed using the experimental data and mathematical models applied to cumulative hydrogen productions.

2.2 Inoculum conditioning and characterization

2.2.1 Real mixed bacteria culture

The seed material used for the evaluation of the biological hydrogen production from a real mixed bacteria culture, was a granular sludge collected from a real scale Upflow Anaerobic Sludge Blanket (UASB) anaerobic digester of a brewery factory located in Padova, Italy.

In this experimental work three specific types of granular sludge were studied. The three samples have been identified as samples “Sludge 2011”, “Sludge 2012” and “Sludge 2013”, which differ in the year of collection, that were 2011, 2012 and 2013, respectively.

The granular sludge used was pre-treated prior to starting the experimental tests as inocula of batch test for H_2 production. This pre-treatment is based on a heating procedure consisting of boiling the sludge sample at a fixed temperature of 100° C for 4 hours, in an oven.

This heat treatment of inoculum was evaluated to be optimal for selecting H_2 producing microorganisms, characterized by high H_2 conversion yields, and for inhibiting of the methanogenic activity (Alibardi *et al*., 2012).

The three samples of the granular sludge used for hydrogen production tests were characterized by analyzing the content of: Total Solids (TS), Volatile Solids (VS), Total Organic Carbon (TOC), Total Kjeldahl Nitrogen (TKN), Ammonium nitrogen and Total Phosphorous, and these results are reported in Table 1. It is important to note that heat treatment did not change the physical-chemical composition of the sludge.

Table 1. Physical and chemical characterization of the granular sludge samples.

Parameter	**Sample Name**		
	Sludge 2011	**Sludge 2012**	**Sludge 2013**
TS±SD [%]	9 ± 1	13 ± 1	10 ± 1
VS±SD [% $_{of\ TS}$]	73 ± 1	72 ± 1	80 ± 1
TOC±SD [% $_{of\ TS}$]	39 ± 1	40 ± 1	45 ± 1
COD±SD [mgCOD/gTS]	1164 ± 5	1248 ± 5	1273 ± 5
TKN±SD [mg-N/g-TS]	67.6 ± 0.5	77.9 ± 0.5	82.0 ± 0.5
NH_4^+±SD [mg-N/g-TS]	29.2 ± 0.5	13.9 ± 0.5	20.2 ± 0.5
P_{tot}±SD [mg-P/g-TS]	19.6 ± 0.5	13.3 ± 0.5	18.0 ± 0.5

2.2.2 Batch mixed reactor

For the purpose to evaluate hydrogen production rates at different F/M ratios (food over microorganism ratio) a system was created, working as a batch reactor. This system was provided of a continuous pH monitoring and controlling system and a continuous biogas production monitoring system.

The seed material used for the evaluation of the biological hydrogen production from a real mixed bacteria culture, was the granular sludge from 2013 (the physical and chemical characterization of this granular sludge is presented in Table 1). This sludge was selected because it showed, in the first part of this work, the best performance in terms of hydrogen production rate. As previously reported, the granular sludge used was pre-treated prior to starting the experimental tests as inocula of batch test for H_2 production.

2.2.3 Pure bacteria culture

In order to work on the utilization of pure cultures for the development of a specific inoculum for hydrogen production from organic substrates, an internal co-operation was established with colleagues from the Department of Agronomy Food Natural Resources Animals and Environment of the University of Padova.

In 2011 an investigation was conducted which aimed at the development of an efficient microbial inoculum for the industrial conversion of organic residues into hydrogen. One hundred and twenty microbial strains, previously isolated from mixed consortia with interesting H_2 fermentative performances from glucose, were genetically identified and screened for their extracellular hydrolytic profile on the main components of the organic fraction of municipal solid waste (OFMSW). In the end few *Bacillus* sp. isolates showed promising hydrolytic capabilities (Favaro *et al*., 2011).

In this sense, the bacteria species that showed more promising hydrolytic capabilities were evaluated in this present work, as pure cultures, for their H_2 production potentials from both simple and complex substrates.

Therefore, will be analysed their main role as inocula of batch test for H_2 production, and then will be evaluated their activity compared with the real mixed bacteria culture activity. The Table 2 shows the microbial strains that were used.

Table 2. Identification of the microbial strains used and their hydrolytic abilities.

Microbial strains used	Microbial sample name	Hydrolytic abilities
***Bacillus licheniformis* LF1.33**	A	Cellulose Hemicellulose Starch Protein
***Paenibacillus cookie* LF2.3**	B	Starch
***Bacillus* sp. LF2.8**	C	Starch Protein
***Paenibacillus* sp. LF4.8**	D	Pectin Protein
***Bacillus farraginis* LF2.7**	E	Starch Protein

Two specific experiments were defined in which the variables were the bacteria species and the substrate used. Each experiment was divided in two sub-parts, named Run 1 and Run 2. The delineation of both experiments is reported in Table 3.

Table 3. Delineation of the experimental activities performed in batch test.

Experiment performed		Microbial sample name	Substrate added	
			Composition	Quantity [g/l]
Experiment Nº 1	**Run 1**	A	Glucose Yeast Extract	5 3
		B	Glucose Yeast Extract	5 3
		C	Glucose Yeast Extract	5 3
	Run 2	A1, B1, C1	----	0
		A2, B2, C2	Glucose	5
		A3, B3, C3	Glucose	10
Experiment Nº 2	**Run 1**	A	Nutrient Broth*	5
		B	Nutrient Broth*	5
		C	Nutrient Broth*	5
		D	Nutrient Broth*	5
		E	Nutrient Broth*	5
	Run 2	A	Glucose	5
		B	Glucose	5
		C	Glucose	5
		D	Glucose	5
		E	Glucose	5

*Nutrient Broth (NB) is composed by: peptone bacteriological (5 g/l); beef extract (1.5 g/l); yeast extract (1.5 g/l); NaCl (5.0 g/l).

2.3 Batch test for hydrogen production

2.3.1 Real mixed bacteria culture

The hydrogen production tests were performed in batch reactors under mesophilic conditions. Batch reactors consist in 0.5 liter Pyrex vessels, hermetically closed by means of a plug with a silicone septum that allows the gas and water sampling with a syringe. The working volume of 250 ml of the reactor is made up of: the substrate, which was glucose, $C_6H_{12}O_6$, (at a concentration of 5 g/l); the seed material (50 g of granular sludge) and the required phosphate buffer solution to set the pH at 5.5, which was the optimum for hydrogen production by mixed anaerobic cultures obtained by many previous studies (Van Ginkel & Sung, 2001; Fan *et al*., 2004). The ratio between the volatile solids of the substrate to be degraded and the volatile solids of the inoculum (food over microorganism ratio – F/M) was set at 0.35 gVS/gVS.

Anaerobic conditions were obtained by making nitrogen flow trough the head space of the vessel for 3 minutes. After this operation the excess pressure was removed in order to re-establish the atmospheric pressure. The mesophilic conditions were guaranteed by keeping the reactors in a water bath at a steady temperature of 35° C (± 1° C).

Each test was carried in triplicate and were made two blank tests of each sample, containing only the respective inoculum and the buffer, that were performed in order to assess the biological hydrogen production of the sole biomass present into the sludge.

The amount of biogas produced was recorded daily, using the water displacement method and biogas composition in terms of hydrogen, carbon dioxide and methane was measured by a gas chromatograph, in this order quantity and the quality of the biogas were measured. Liquid samples were collected at the end of the fermentation tests and analyzed for the concentration of volatile fatty acids. These tests lasted 7 days and also during this period the pH value of the digestion liquid was monitored.

2.3.2 Batch mixed reactor

The hydrogen production tests were performed in batch reactors under mesophilic conditions that were integrated in a circuit between a pH control and monitoring system and a bascule.

As the time that the biogas is formed it is directed to a channel that ends inside a wet tip gas meter, which is inserted inside a container with water. Over time, the gas meter is filled with a volume of biogas produced by the batch reactor. When 3.8 ml of biogas remains inside the gas meter it moves and consequently the biogas is released out of the system. This movement is shown automatically in the system and so, it is possible to know at the end of the batch test the volume of biogas formed by the number of movements performed by the wet tip gas meter.

Simultaneously there is a system for the pH control and monitoring, which makes possible to maintain the more favorable pH value for the hydrogen production which is, in this case, 5.5. This system is directly connected to the batch reactor by a pH meter, which is connect to a device which triggers the introduction of sodium hydroxide (NaOH), through a needle inserted into the batch reactor. Thus, whenever the pH value decreases it is automatically introduced, in the reactor, a certain amount of NaOH to achieve the pH value established as optimal.

For the purpose to evaluate hydrogen production rates at different F/M ratios (food over microorganism ratio) three different batch tests were created. The following F/M ratios were tested: 0.39 gVS/gVS; 0.78 gVS/gVS and 1.56 gVS/gVS. Each test were named as “S_40”, “S_20” and “S_10” respectively. In these different tests were used batch reactors for each of the four experiments, which consist in 0.5 liter Pyrex vessels, hermetically closed by means of a plug with a silicone septum that allows the gas and water sampling with a syringe. The reactor performance of each experiments was assessed at four different quantities in granular sludge and so the working volume of 250 ml of the reactor is made up of: the substrate, which was glucose ($C_6H_{12}O_6$) at 5 g/l as concentration in all the four test; the seed material (granular sludge) with an amount of 40 g, 20 g and 10 g, for each test respectively; distilled water and sulfuric acid (H_2SO_4) with 0.1 M, to set the initial pH at 5.5.

Anaerobic conditions were obtained by making nitrogen flow through the head space of the vessel for 3 minutes. The mesophilic conditions were guaranteed by placing the reactor in contact with a heating system programmed to keep the temperature at 35 °C (± 1° C).

The amount of biogas produced, the pH variation and the NaOH added were recorded at the end of each batch test, with the aid of a computer program, which is connected to the batch system enabling the capture of a photograph, from 10 to 10 minutes, with the information referred before.

When the fermentation test ends, the biogas composition in terms of hydrogen, carbon dioxide and methane was measured by a gas chromatograph. Then a liquid sample was collected and it was analyzed for the concentration of VFAs, Ammonium nitrogen and Total Phosphorous and Titration at 7 points.

2.3.3 Pure bacteria culture

2.3.3.1 Experiment nº1

As previously reported this experimental phase was divided in two parts, which were nominated as “Run 1” and “Run 2”.

In “Run 1” batch reactors, 0.5 liter Pyrex vessels were filled with 250 ml of hydrogen production medium (HPM) containing glucose (5 g/l) and yeast extract (3 g/l) and the required buffer solution, which was a phosphate buffer solution to set the pH at 5.5.

Each microbial strain was aerobically pre-grown in 200 mL Erlenmeyer flasks containing 50 mL of HPM or Nutrient Broth (N.B) (2.5, 5, 7.5, 10 %, v/v). The growth of each strain, inoculated at an initial optical density (OD 600nm) value of 0.06, was monitored by determining the OD at 600 nm with a spectrophotometer (Ultrospec 2000, Pharmacia Biotech).

After aseptically inoculated, the reactors were hermetically closed using a silicon plug. Anaerobic conditions were obtained by making nitrogen flow trough the head space of the vessel for 3 minutes. After this operation the excess pressure was removed in order to re-establish the atmospheric pressure.

The mesophilic conditions were guaranteed by keeping the reactors in a water bath at a steady temperature of 35° C (± 1° C).

Each test was carried in triplicate and was made one blank test, named as Non – Inoculated (NI) sample, containing only the HPM solution and the buffer. The blank test was performed in order to be sure that the sterilized conditions are maintained.

The amount of biogas produced was recorded daily, using the water displacement method and biogas composition in terms of hydrogen, carbon dioxide and methane was measured by a gas chromatograph, in this order the quantity and the quality of the biogas were measured. Liquid samples were collected at the second day and at the end of the fermentation tests and analyzed for the concentration of volatile fatty acids.

These tests took place over 5 days, after which no longer significant production of hydrogen was noted. Also during this period the pH value of the digestion liquid and the OD were monitored.

In the end of the "Run 1", residual glucose in the HPM medium was measured according to the dinitrosalicylic (DNS) method described by Miller (1959).

In "Run 2" was analyzed the behavior of each sample introducing different quality and quantity of substrate. In this sense, it was done the following procedure: in samples identified with the number 1 (A1, B1 and C1) the initial conditions were maintained and so these samples were used as blank samples in "Run 2"; 5 g/l of glucose was added in samples identified with the number 2 (A2, B2 and C2); 10 g/l of glucose was added in samples identified with the number 3 (A3, B3 and C3).

The batch reactors, 0.5 liter Pyrex vessels, were filled with the amount of subtract planed for each sample and the required buffer solution, which was a phosphate buffer solution to set the pH at 5.5. After inoculation, all the procedure followed it is the same as previously reported.

The amount of biogas produced was recorded daily, using the water displacement method and biogas composition in terms of hydrogen, carbon dioxide and methane was measured by a gas chromatograph, in this order the quantity and the quality of the biogas were measured. Liquid samples were collected at the end of the fermentation tests and analyzed for the concentration of volatile fatty acids.

These tests took place over 3 days, after which no longer significant production of hydrogen was noted. Also during this period the pH value of the digestion liquid and the optical density was monitored.

2.3.3.2 Experiment nº2

This experimental work was divided in two parts, which were nominated as "Run 1" and "Run 2".

In "Run 1" batch reactors, 0.5 liter Pyrex vessels, were filled with Nutrient Broth (NB) and a phosphate buffer solution to set the pH at 5.5. After inoculation, all the procedure followed was the same as previously reported. Each test was carried in triplicate and in this experiment were made two blank tests, named as NB1 and NB2, containing only the Nutrient Broth solution and the buffer. The blank test was performed in order to be sure that the sterilized conditions are maintained.

The amount of biogas produced was recorded daily, using the water displacement method. Biogas composition in terms of hydrogen, carbon dioxide and methane was measured by a gas chromatograph, in this order the quantity and the quality of the biogas were measured. Liquid samples were collected at the end of the fermentation tests and analyzed for the concentration of volatile fatty acids. These tests took place over 12 days, after which no longer significant production of hydrogen was noted. Also during this period the pH value of the digestion liquid and the optical density was monitored.

In "Run 2" it was analyzed the behavior of each sample introducing different quantity of substrate. In this sense, it was added glucose (5 g/l) in all the samples. The batch reactors, 0.5 liter Pyrex vessels, were filled with the amount of subtract planed for the samples and the required buffer solution, which was a phosphate buffer solution to set the pH at 5.5. After inoculation, all the procedure followed it is the same as previously reported.

The amount of biogas produced was measured in exactly the same manner as the "Run 1". Liquid samples were collected at the second day and at the end of the fermentation tests and analyzed for the concentration of volatile fatty acids.

These tests took place over 7 days, after which no longer significant production of hydrogen was noted. Also during this period the pH value of the digestion liquid and the optical density was monitored.

2.4 Methods

2.4.1 Analytical methods

Total Solids (TS), Volatile Solids (VS), Total Kjeldahl Nitrogen (TKN), Ammonium nitrogen and Total Phosphorous were analysed according to Standard Methods (APHA, 1999). Total Organic Carbon (TOC) was quantified using a Total Carbon Analyzer (TOC – V CSN, Shimadzu).

Volatile Fatty Acids (VFAs) concentrations were analyzed using a gas chromatograph (Varian 3800) equipped with flame ionization Detector (FID), Stabilwax – DA column, nitrogen as carrier gas.

The composition of biogas in the headspace was measured by means using a micro-GC (Varian 490-GC) equipped with a 10-meter MS5A column and a 10-meter PPU column. Helium was used as carrier gas.

To analyze the Optical Density of the microorganisms inside the batch reactors it was necessary collect 3 ml of each sample and measured it, using a spectrofotometer. Subsequently the same samples were used to determine their pH, with the aid of a pH meter.

2.4.2 Experimental data results

The amount of biogas produced by fermentation was measured by means of the dislocation method. According to the functional principle of dislocation, the excessive pressure formed into the head space of reactors moves a volume of liquid, present in another bottle, equal to the volume of the biogas that was produced by fermentation. The displaced liquid is an acid saline solution (pH < 3 and 25% NaCl), where carbon dioxide (CO_2) and methane (CH_4) do not dissolve into, and it is collected in a granular cylinder.

The volume of hydrogen produced in the period of between two measurements, *t* and (*t-1*), has been calculated according to the following equation (1) (Van Ginkel *et al*., 2005):

$$V_{C,t} = C_{C,t}\ V_{G,t} + V_H\ (C_{C,t} - C_{C,t-1}) \qquad (1)$$

where:

$V_{C,t}$ is the volume of H_2 produced in the period of time between time *t* and time *t-1*;

$C_{C,t}$ is the concentration of hydrogen measured at time *t*;

$V_{G,t}$ is the volume of biogas produced in the period of time between time *t* and time *t-1*;

V_H is the volume of the reactor headspace;

$C_{C,t-1}$ is the concentration of hydrogen measured at time *t-1*.

2.4.3 Mathematical models of hydrogen production

To compare the results obtained from the batch tests, data were interpolated using a Gompertz equation when dealing with a latency phase or a first order kinetics equation in a situation of exponential production.

The Gompertz equation (2) used is as follows (Lay *et al*., 1997):

$$B(t) = B_0 \cdot exp\left\{- exp\left[R \cdot e\frac{(\lambda - t)}{B_0}\right] + 1\right\} \qquad (2)$$

where:

B(t) is the cumulative biogas/hydrogen production at time t(d) (Nml/gVS);

B_0 is the maximum biogas/hydrogen production (Nml/gVS);

R is the biogas/hydrogen production rate (Nml/gVS.d);

λ is the latency phase (d);

e is Euler's number.

Average values of cumulative biogas and hydrogen production from each experimental condition were used to obtain the values of the parameters B_0, R and λ. These parameters were estimated by minimizing the sum square of errors between experimental data and results from the model. The estimations were carried out by using the "Solver" function in Excel of Microsoft Office.

When no latency phase was detected, the data of hydrogen production were interpolated using an exponential function (Trzcinski & Stuckey, 2012), as it is showed in the first order kinetics equation bellow (3):

$$P(t) = P_0 \cdot (1 - e^{-kt}) \quad (3)$$

where:

$P(t)$ is the cumulative biogas/hydrogen production at time t(d) (Nml/gVS);

P_0 is the maximum biogas/hydrogen production (Nml/gVS);

k is the kinetics degradation constant (d^{-1}).

Average values of cumulative biogas and hydrogen production from each experimental condition were used to obtain the values of the parameters P_0 and k. These parameters were estimated by minimizing the sum square of errors between experimental data and results from the model. The estimations were carried out by using the "Solver" function in Excel of Microsoft Office.

It is important to note that data of hydrogen yield are expressed as ml of hydrogen at temperature of 0 °C and pressure of 1 atm.

3. RESULTS AND DISCUSSION

3.1 Biological hydrogen potential production

3.1.1 Real mixed bacteria culture

With the purpose to evaluate the biological hydrogen production potentials from a real mixed bacteria culture, granular sludge samples, collected at different times from a real scale plant, were used as inocula for this experiment.

The results of hydrogen production potentials are reported in Table 4. The mathematical model parameters, obtained from the average cumulative hydrogen productions of the experimental data, are described in Table 5 and the cumulative hydrogen productions curves from experimental results and from mathematical models obtained by the samples "Sludge 11", "Sludge 12" and "Sludge 13" are showed in Figure 1. In the Figure 2 it is possible to simultaneously compare the results obtained from the three samples.

Table 4. Results from hydrogen production batch tests, obtained by each real mixed bacteria culture sample.

Sample name	Hydrogen yield±SD [Nml H_2 / gVS]	Volatile Fatty Acids			
		Acetic acid [mg/l]	*Propionic acid [mg/l]*	*Butyric acid [mg/l]*	*Isovaleric acid [mg/l]*
Sludge 2011	113.2 ± 20.5	221.3	40.2	45.3	35.6
Sludge 2012	119.6 ± 18.3	774.9	44.4	775.2	49.5
Sludge 2013	148.2 ± 1.2	806.1	46.7	807.2	112.3

Table 5. Mathematical model parameters, obtained by each real mixed bacteria culture sample.

Sample name	Exponential function parameters		
	P_0 [Nml H_2 / gVS]	k [d^{-1}]	Max rate [(Nml H_2 / gVS)*d^{-1}]
Sludge 2011	113.2	3.0	339.6
Sludge 2012	119.6	2.9	346.8
Sludge 2013	148.2	7.3	1081.9

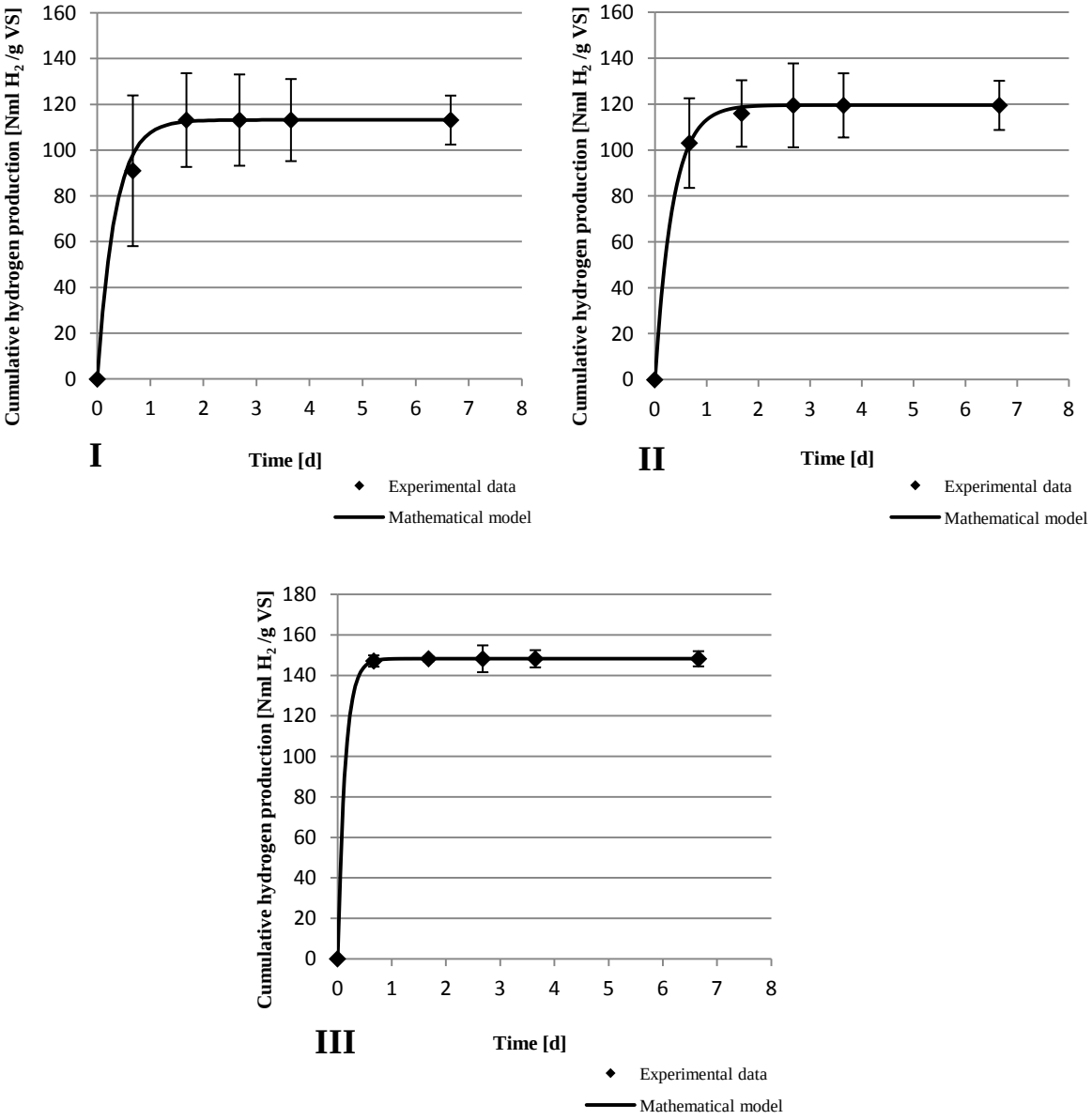

Figure 1. Cumulative hydrogen productions from average experimental data and from the mathematical model by the samples: (I) "Sludge 2011"; (II) "Sludge 2012"; (III) "Sludge 2013". The vertical bars over the experimental data represent the standard deviations of the triplicate.

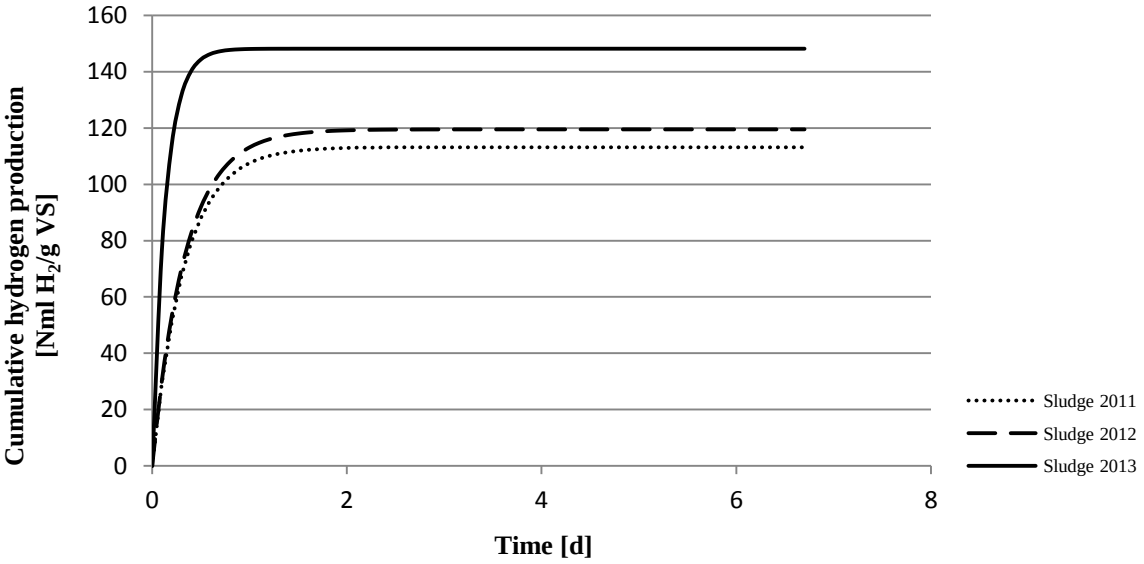

Figure 2. Comparison between the cumulative hydrogen productions from the mathematical model, by the samples "Sludge 2011", "Sludge 2012" and "Sludge 2013".

Methane was not detected, because of elimination of methane producers by heat digestion of sludge. The highest hydrogen production was measured for the sample "Sludge 2013", with a total hydrogen production of 148.2 $NmlH_2/gVS$, whereas the sample "Sludge 2011" showed the lowest production, with a total hydrogen production of 113.2 $NmlH_2/gVS$.

According to the results, it is possible to conclude that the age of the sludge is directly associated to the behavior of the bacteria, that are contained in the inoculum, and so, as more recent is the granular sludge, higher hydrogen production will be achieved and higher will also be the rate process.

The operational pH value is considered to be a crucial parameter during the fermentation process, because it affects the hydrogenase activity and the metabolism pathway (Vijayaraghavan & Soom, 2004). During the experimental activities the pH of all the samples remained practically constant, ranging from 5.5 and 6.0, and subsequently this parameter contributed to the achievement of high yields of hydrogen.

In order to identify the constraints of the dark fermentation process on the hydrogen production, further analyses were done on the liquid samples to know the VFAs which were formed during the fermentation process.

When bacteria metabolize simple sugars, such as glucose, organic acids are produced as sub-products, since there is an incomplete conversion of the substrate to carbon dioxide (Van Ginkel *et al*., 2005). The following equations show the production of these small organic acids in two different ways. In one way, glucose in biomass gives a maximum yield of 4 H_2 per glucose when acetic acid is the by-product (Hawkes *et al*., 2002):

$$C_6H_{12}O_6 + 2\ H_2O \rightarrow 2\ CH_3COOH + 2\ CO_2 + 4\ H_2\ (4\ ATP) \qquad (3)$$

In other way, half of this yield per glucose is obtained with butyrate as the fermentation end product (Hawkes *et al*., 2002):

$$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2\ CO_2 + 2\ H_2\ (3ATP) \qquad (4)$$

Until now it was not known why one way is favored over another, however the combination of both is observed in all bacteria populations (Van Ginkel *et al*., 2005).

It is important to note that the overall equation for the production of propionate from glucose, shows that this involves the consumption of H_2 (Vavilin *et al*., 1995):

$$C_6H_{12}O_6 + 2\ H_2 \rightarrow 2\ CH_3CH_2COOH + 2\ H_2O \qquad (5)$$

Thus the production of propionate should be avoided. Vavilin *et al*. (1995) stated that the limiting substrate for butyrate production is glucose, while the limiting substrate for propionate production is H_2, and the two groups of organisms producing these end products are in balance in the microbial consortium producing H_2. Limiting the amount of propionate-formers by heat treatment of the inoculum may aid in biasing the community towards butyrate production.

Figure 3 presents the VFA composition formed by the bacteria during the fermentation process.

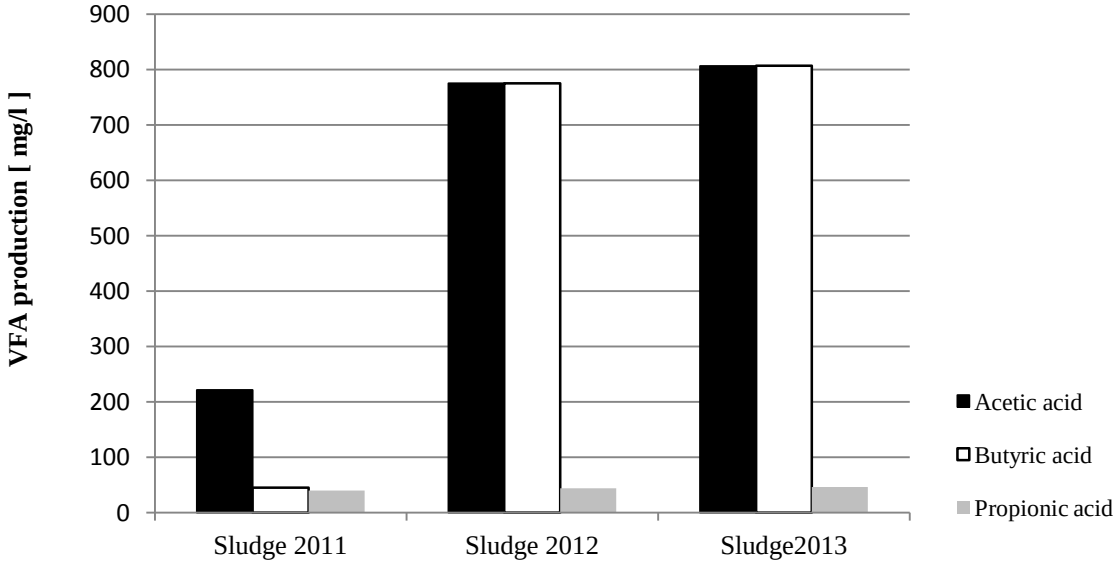

Figure 3. Volatile fatty acids composition of dark fermentation the samples "Sludge 2011", "Sludge 2012" and "Sludge 2013".

The previous results show that "Sludge 2013" had the higher acetate concentration compared to "Sludge 2011" and "Sludge 2012". These results agreed with the data on hydrogen production.

It is also important to note that batch tests were conducted over 6 days during which there was no addition of substrate and the initial conditions, which were the optimal, tend to be altered. Such factors have as consequence an ecological bacteria selection due to the limitation of "food" at "unfavorable conditions" and so a sharp reduction in the number of bacteria occurs.

Furthermore, no lag phase was registered and consequently the biomass did not suffer inhibition with the imposed substrate concentration and its adaption was not necessary in this conditions (Maintinguer *et al*., 2008).

After the peak value of hydrogen production a phase of hydrogen consumption was noted. These results can be seen in the Figure 4. In the Figure 5 it is possible to simultaneously compare these results obtained from the three samples. Furthermore, Table 6 shows the consumption rate of each sample.

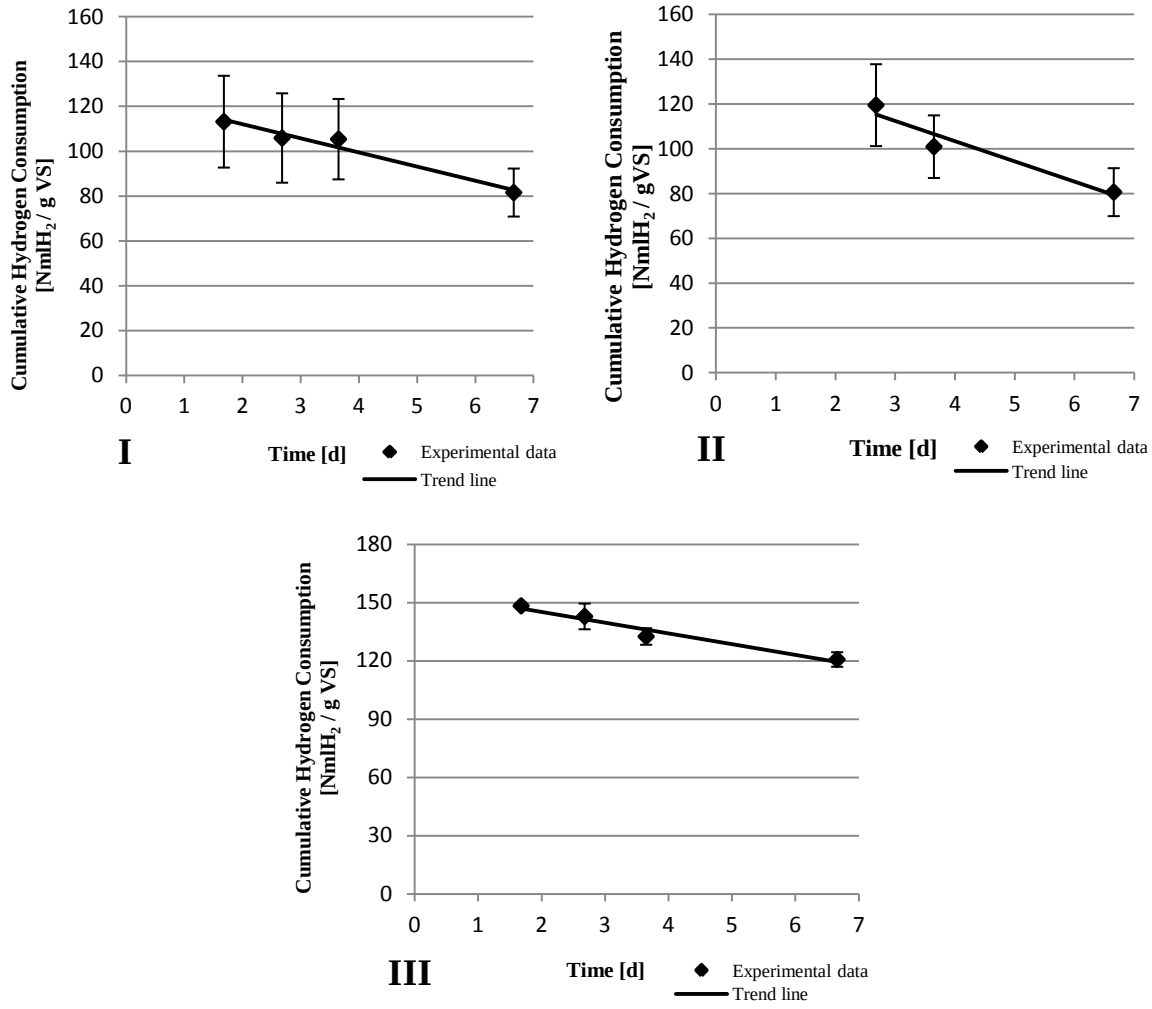

Figure 4. Cumulative hydrogen consumption from average experimental data by the samples: (I) "Sludge 2011"; (II) "Sludge 2012"; (III) "Sludge 2013". The vertical bars over the experimental data represent the standard deviations of the triplicate.

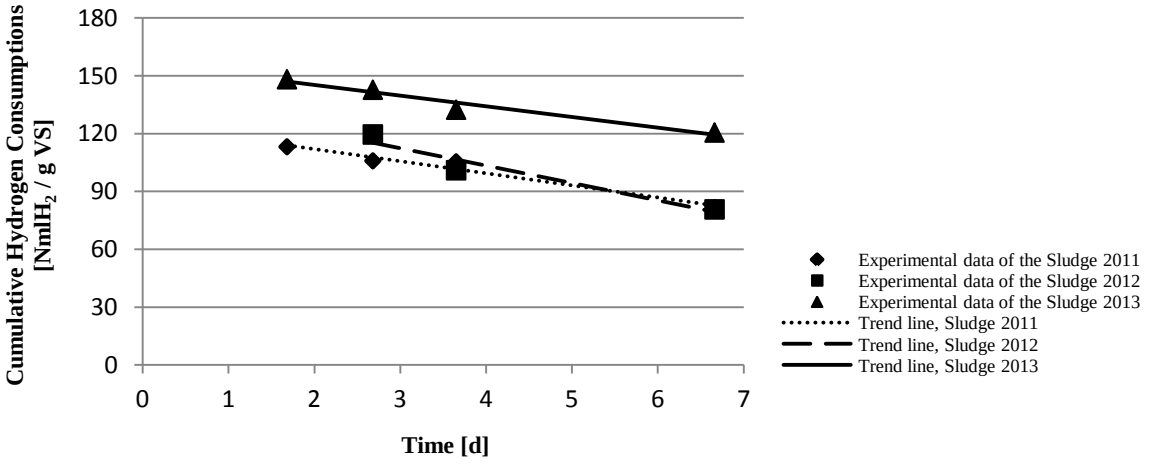

Figure 5. Comparison between the cumulative hydrogen consumptions, by the samples "Sludge 2011", "Sludge 2012 and "Sludge 2013", and their trend lines.

Table 6. Results from hydrogen consumption batch tests, obtained by each real mixed bacteria culture sample.

Sample name	Hydrogen consumption ± SD [Nml H_2 / gVS]	Time [d]	Rate [(Nml H_2) / (gVS*d)]
Sludge 2011	31.6 ± 10.7	5.0	6.3
Sludge 2012	38.9 ± 10.7	4.0	9.7
Sludge 2013	27.6 ± 3.7	5.0	5.5

The previously graphs allows to understand that sample "Sludge 2013" had the lower consumption rate, which was 5.5 Nml H_2/(gVS*d) , in turn the sample "Sludge 2012" showed the highest consumption rate equal to 9.7 Nml H_2/(gVS*d).

These results mean that the sample "Sludge 2013", which is the most recent, provides the best global conditions since it shows the highest hydrogen production, as well as the lowest rate of hydrogen consumption.

The hydrogen consumption is a phenomenon that presumably occurs because homoacetigenic bacteria consume hydrogen to produce acetic acid and hydrogenases bacteria recycle a portion of hydrogen produced (Hellenbeck & Benemann, 2002).

Homoacetogenic bacteria are strictly anaerobic microorganisms which catalyze the formation of acetate from H_2 and CO_2. Unfortunately, the pretreatment of the inoculum by heating to select spore-forming bacteria is not suitable for inhibiting of homoacetogenic bacteria (Guo *et al*., 2010).

Thus, the reasons that lead to a better performance of the sample nominated as "Sludge 2013", comparatively to the other samples, are: efficacy of the heat treatment, by selecting H_2 producing microorganisms and inhibiting methanogenic activity; higher number of fermentative bacteria after the heat-treatment of the inoculum and lower number in hydrogen consumers microorganisms, as homoacetogenic bacteria, that compose the inoculum.

3.1.2 Batch mixed reactor

For the purpose to evaluate hydrogen production rates at different F/M ratios (food over microorganism ratio) a system was created, working as a batch reactor. In this sense, three different batch tests, which were named as "S_40", "S_20" and "S_10", were studied. The results of the three tests can be seen in the Figure 6. Moreover, Table 7 shows the detailed results of the experimental data, using Gompertz equation as mathematical model and in Table 8 are represented the analytical analyses, by each sample.

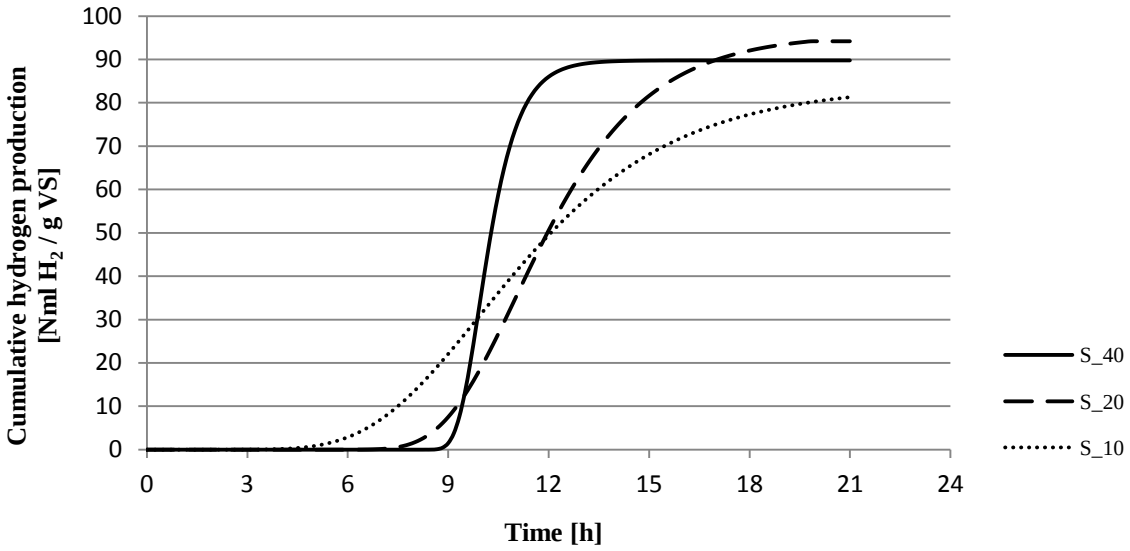

Figure 6. Comparison between the hydrogen production rates, by the samples "S_40", "S_20" and "S_10".

Table 7. Results from hydrogen yields and rates at different F/M ratios, from the mathematical model, by each sample using the batch mixed reactor.

Sample name	Inoculum added [g]	Hydrogen yields [Nml H_2 / gVS]	Rate [(Nml H_2) / (gVS*d)]	λ [d]
S_40	40.0	88.9	6.3	0.39
S_20	20.0	91.2	9.7	0.37
S_10	10.0	77.5	5.5	0.28

Table 8. Results from analytical analysis, by each sample using the batch mixed reactor.

Sample name	Ammonium Nitrogen [mgN/l]	Total Phosphorous [mgP/l]	Total Alcalinity [mg/l as $CaCO_3$]	Volatile Fatty Acids			
				Acetic acid [mg/l]	*Propionic acid [mg/l]*	*Butyric acid [mg/l]*	*Isovaleric acid [mg/l]*
S_40	161.0	107.7	5993.4	276.0	21.0	257.0	< 10
S_20	68.6	53.6	2289.1	226.0	< 10	173.0	< 10
S_10	16.8	21.0	1653.9	172.0	< 10	65.3	< 10

The main objective of this experiment is to verify the relation between the amount of inoculum added and its rate of hydrogen production, as well the hydrogen yields and so the results allow to understand that:

- The behavior of each sample, in terms of analytical analysis, is similar taking into account the amount of inoculum added in each test, as the results in Table 8 show;

- The hydrogen produced is independent of the amount of inoculum added, since all tests achieved approximately the same amount of hydrogen yields;

- On the contrary, the quantity of inoculum added is responsible on the larger or smaller duration of the lag phase, as well as the rate of hydrogen production. Since all the tests showed different performances with regard to these parameters.

3.1.3 Pure bacteria culture

Enhancing the hydrogen production efficiency is one of the major challenges to dark hydrogen fermentation. To achieve such a purpose, numerous research studies on anaerobic microbes have been intensively developed in recent years, and some new or efficient bacterial species and strains for dark hydrogen fermentation have been isolated and recognized (Fang *et al*., 2002).

Therefore, the research direction is to improving both hydrogen yield and hydrogen production rate simultaneously. In order to yield as much hydrogen as possible, it has to be created one optimal microbial metabolism.

In this sense, this part of the study will investigate the hydrogen production potential of five dark fermentative bacteria (*Bacillus licheniformis*, *Paenibacillus cookie*, *Bacillus* sp., *Paenibacillus* sp., *Bacillus farraginis*) using glucose as substrate, available in different concentrations, under anaerobic conditions. Will also be compared the hydrogen production potential using Nutrient Broth (NB) as substrate.

3.1.3.1 Experiment nº 1: "Run 1"

In this experiment glucose was used as substrate, with a concentration of 5 g/l, in each sample. The results of hydrogen production potentials are reported in Table 9. Even more the mathematical model parameters, obtained from the average cumulative hydrogen productions of the experimental data, are described in Table 10.

The cumulative hydrogen productions curves, from mathematical models, and the OD and pH values obtained by the samples A, B and C are showed in Figure 7.

Table 9. Results from hydrogen production batch tests, for the experiment nº1–"Run 1".

Sample name	**Hydrogen yield±SD [Nml H_2 / gVS]**	**Volatile Fatty Acids**			
		Acetic acid [mg/l]	*Propionic acid [mg/l]*	*Butyric acid [mg/l]*	*Isovaleric acid [mg/l]*
A	9.5 ± 2.0	103.6	8.4	1.0	1.9
B	40.7 ± 30.2	91.5	3.1	8.4	3.1
C	10.8 ± 2.6	111.8	7.8	1.52	5.3

Table 10. Mathematical model parameters, obtained by the sampled from the experiment nº1 – "Run 1".

Sample name	**Parameters of the Gompertz equation**		
	B_0 [Nml H_2 / gVS]	λ [d]	R [(Nml H_2) / (gVS*d)]
A	9.6	2.7	9.9
B	41.9	1.9	23.3
C	10.8	2.2	14.0

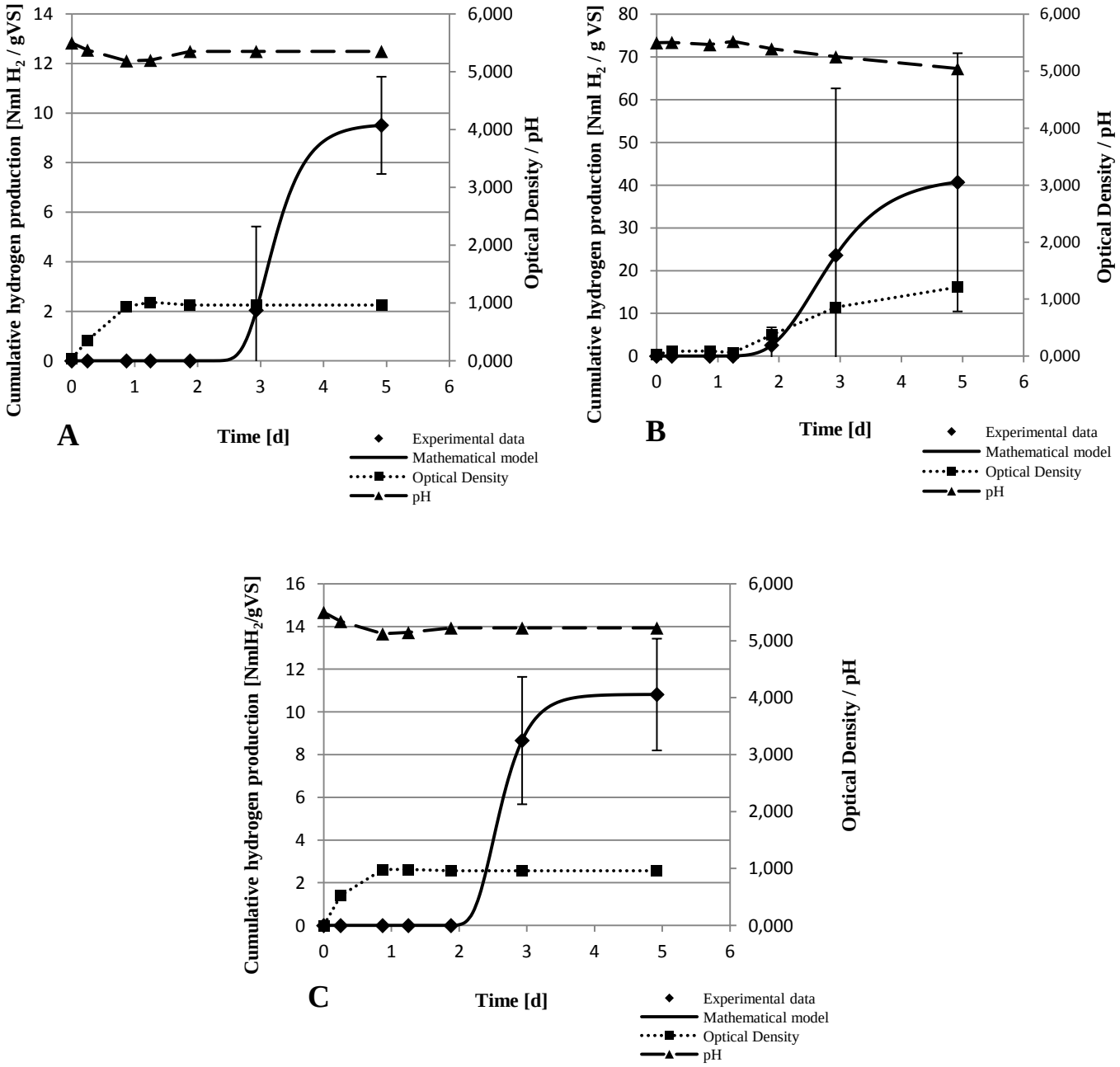

Figure 7. Optical Density and pH variations, over time, and cumulative hydrogen productions from average experimental data and from the mathematical model by the samples: (A) *B. licheniformis*; (B) *P. cookie*; (C) *Bacillus* sp. The vertical bars over the experimental data represent the standard deviations of the triplicate.

The highest hydrogen production was measured for *P. cookie* (sample B), with a total hydrogen production of 41.9 $NmlH_2/gVS$, in turn. *Bacillus* sp. (sample C) showed a total hydrogen production of 10.8 $NmlH_2/gVS$, whereas *B. licheniformis* (sample A) showed the lowest production, with a total hydrogen production of 9.6 $NmlH_2/gVS$.

The previously graphs show that the behavior of *B. licheniformis* and of *Bacillus* sp. are very similar. However *P. cookie* shows a distinct behavior compared to the other two samples.

The species *B. licheniformis* and *Bacillus* sp. show initially a clear rise in the microbial density accompanied by a decrease of pH. Approximately after 1 day is evident an evolution in the volume of fermentative bacteria present in the samples, corresponding to a maximum value of 1.014 in sample *B. licheniformis* , and a maximum value of 0.985 in sample *Bacillus* sp.

Regarding specie *P. cookie*, this shows a more gradual behavior, in respect of the increase of the number of fermentative bacteria and the decrease of the pH. A maximum value of 1.212 was obtained, practically at the end of 5 days. However, in this case, the maximum OD value obtained is practically the same as in *B. licheniformis* and in *Bacillus* sp.

Actually, carbohydrates are the preferred organic carbon source for hydrogen-producing fermentations (Hawkes *et al*., 2002). Thus, and according to the obtained results, it is apparent that microbial species present in each sample consume the glucose, previously added, leading to an increase in OD value. When the carbon source is no longer available, the OD value decreases, remaining constant thereafter.

Furthermore, it is noted an existence of a lag phase in all the samples. This means that the biomass suffered inhibition with the imposed substrate concentration and consequently a period of adaptation to new environmental conditions occurred. In fact, the lag phase can be related to the hydrogen productions, since *P. cookie* showed a lag phase with the lowest period and the highest hydrogen production, contrary to what occurred with *B. licheniformis*.

Initial pH influences the extent of lag phase in batch hydrogen production. Composition of the substrate, media composition, temperature and the type of microbial culture are as well important parameters affecting the duration of lag phase (Kapdan & Kargi, 2006).

As previously mentioned, it is possible to observe that there was a variation of the pH in all the samples. In fact, it decreases evidently during the first phase for *B. licheniformis* and *Bacillus* sp., and in opposite, for *P. cookie*, it decreases in the exponential phase of the hydrogen production. In this sense, can be stated that the decrease in its value, due to production of organic acids, depletes the buffering capacity of the medium resulting in a lower final pH (Patel *et al*., 2012).

3.1.3.2 Experiment nº 1: "Run 2"

In this experiment glucose was used as substrate, with a concentration of 5 g/l (in samples A2, B2 and C2) and a concentration of 10 g/l (in samples A3, B3 and C3). The results of hydrogen production potentials are reported in Table 11. Even more the mathematical model parameters, obtained from the average cumulative hydrogen productions of the experimental data, are described in Table 12.

The cumulative hydrogen productions curves, from mathematical models, and the OD and pH values obtained by the samples A2 and A3, B2 and B2 and C2 and C3 are showed in Figure 8, 9 and 10, respectively. In the Figure 11 it is possible to simultaneously compare the results obtained from all the samples with different quantity in substrate.

Table 11. Results from hydrogen production batch tests, for the samples A2, B2 and C2 and for the samples A3, B3 and C3, in the experiment nº 1 – "Run 2".

Sample name	**Hydrogen yield [Nml H_2 / gVS]**	**Volatile Fatty Acids**			
		Acetic acid [mg/l]	*Propionic acid [mg/l]*	*Butyric acid [mg/l]*	*Isovaleric acid [mg/l]*
A2	14.8	209.8	4.4	6.6	3.2
B2	27.9	129.3	2.1	5.4	3.7
C2	24.0	69.9	7.8	105.3	4.6
A3	2.7	97.0	11.2	11.5	1.6
B3	29.4	55.5	4.1	1.0	3.0
C3	9.9	199.8	7.5	6.3	5.4

Table 12. Mathematical model parameters, for the sample A2, B2 and C2 and for the samples A3, B3 and C3, in the experiment nº 1 – "Run 2".

Sample name	Parameters of the Gompertz equation		
	B_0 [Nml H_2 / gVS]	λ [d]	R [(Nml H_2) / (gVS*d)]
A2	15.1	0.2	23.3
B2	26.8	0.1	50.6
C2	24.6	0.2	20.2
A3	2.7	0.3	2.9
B3	29.5	0.4	70.7
C3	9.7	0.2	15.6

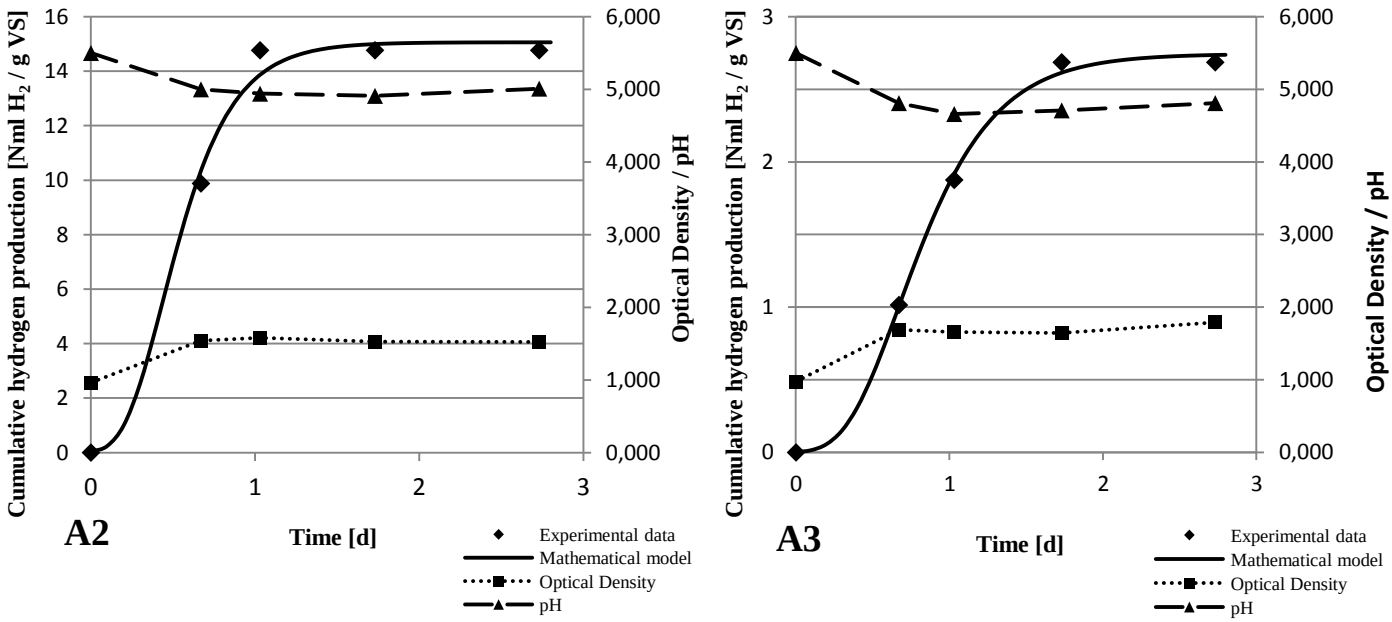

Figure 8. Comparison between the cumulative hydrogen productions from the mathematical model, obtained by the samples A2 and A3.

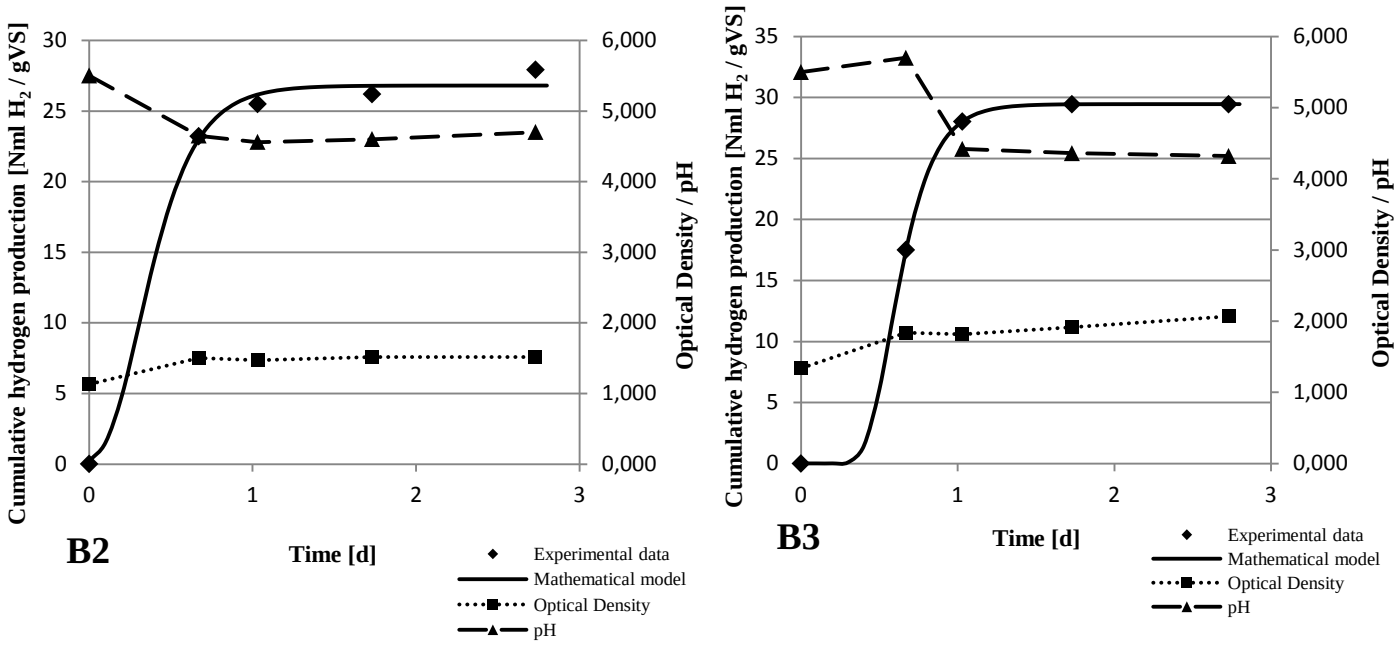

Figure 9. Comparison between the cumulative hydrogen productions from the mathematical model, obtained by the samples B2 and B3.

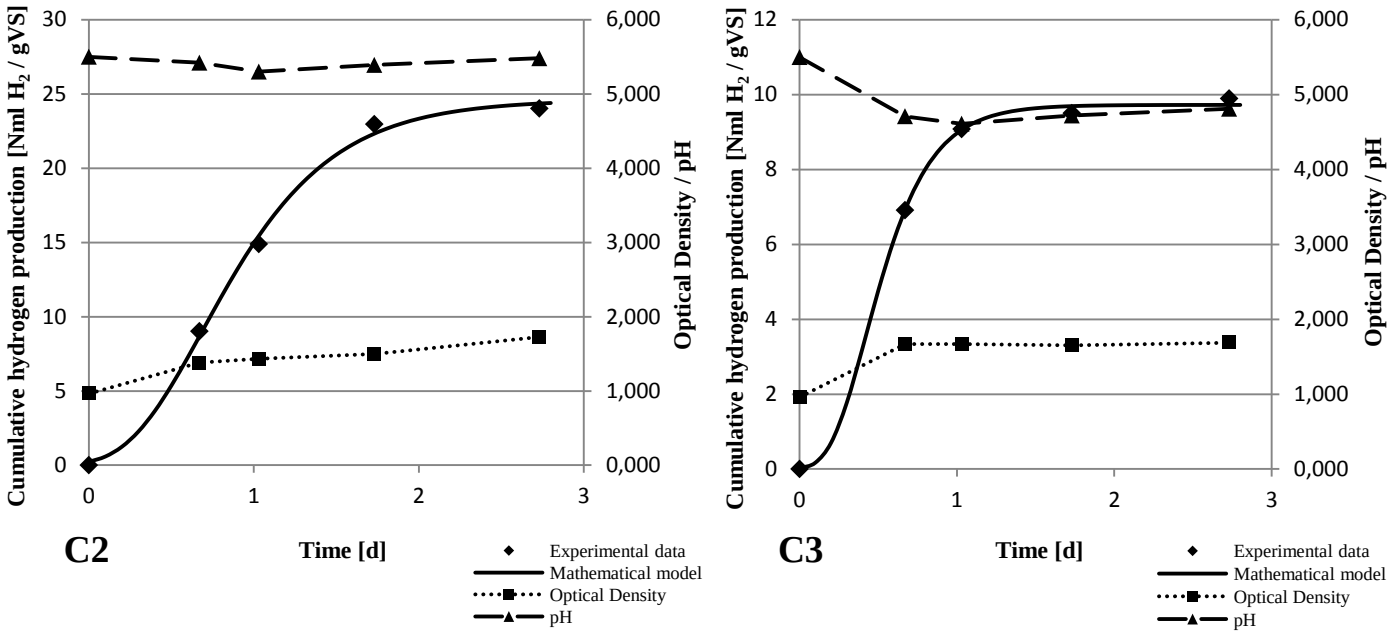

Figure 10. Comparison between the cumulative hydrogen productions from the mathematical model, obtained by the samples C2 and C3.

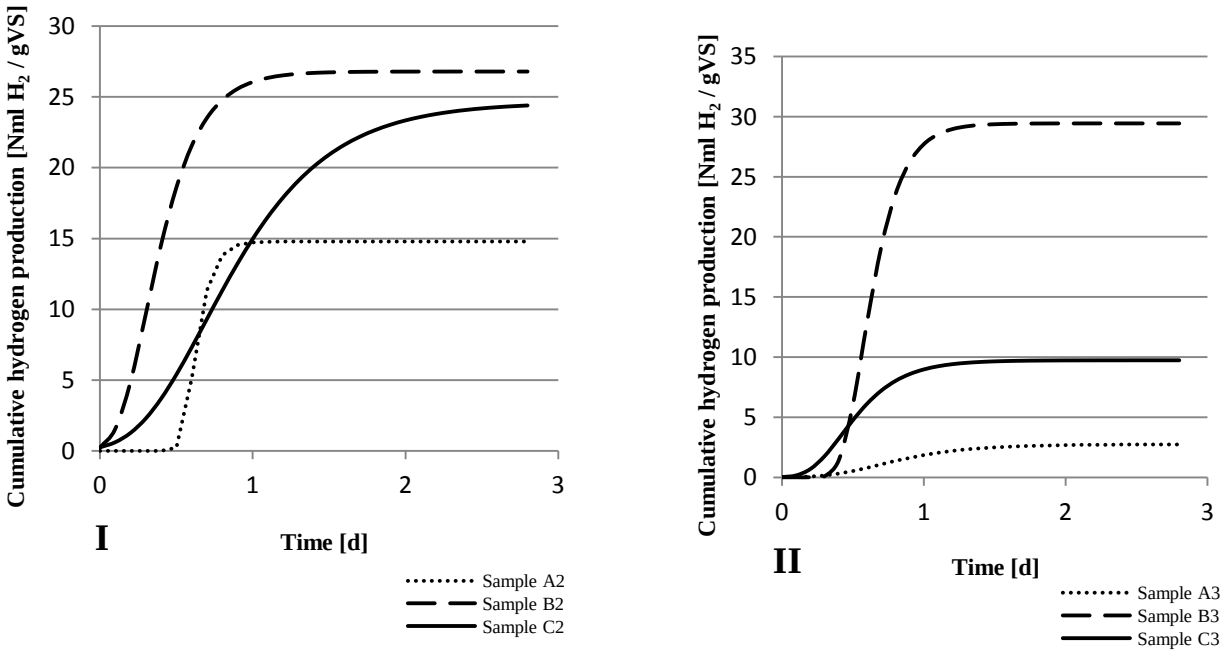

Figure 11. Comparison between the cumulative hydrogen productions from the mathematical model, obtained by the samples A2, B2 and C2 and by the samples A3, B3 and C3, with: (I) 5 g/l of glucose; (II) 10 g/l of glucose.

It is possible to observe that during the early stage, without lag phase, the initial glucose concentrations had less impact on hydrogen production and so cellular growth increased. After this period, the hydrogen production increased rapidly, until reaching a maximum value of hydrogen production, from which the production practically ceased. During the exponential phase of hydrogen production, pH was gradually decreased.

In this sense, the main differences between the samples examined lies in the maximum production rate, given by the parameter *R* in the Gompertz equation, and also in the maximum production yield, (see Table 12).

It was observed that at low glucose concentration (5 g/l), the cumulative hydrogen production was 26.8 $NmlH_2/gVS$ for *P. cookie* (sample B2), while *B. licheniformis* (sample A2) produced 15.1 $NmlH_2/gVS$.

By increasing the glucose concentration to double (10 g/l), the maximum hydrogen production was observed, as before, for *P. cookie* (sample B3), with a production of 29.5 $NmlH_2/gVS$ (value quite similar to the previously result). On the contrary, the species *B. licheniformis* (sample A3) and *Bacillus* sp. (sample C3) followed opposite

tendency, showing a production of 2.7 $NmlH_2/gVS$ and a production of 9.7 $NmlH_2/gVS$, respectively. These results are much lower than the results obtained with a glucose concentration at 5 g/l.

Therefore, the maximum values of hydrogen production were recorded when the glucose concentration was set to 5 g/l, suggesting that high glucose concentration could inhibit the fermentation process.

3.1.3.3 Experiment nº 2: "Run 1"

In this experiment Nutrient Broth (NB) was used as substrate, with a concentration of 5 g/l, in each sample. NB is composed by: peptone bacteriological (5 g/l); beef extract (1.5 g/l); yeast extract (1.5 g/l); NaCl (5.0 g/l).

The results of hydrogen production potentials are reported in Table 13. Even more the mathematical model parameters, obtained from the average cumulative hydrogen productions of the experimental data, are described in Table 14.

The cumulative hydrogen productions curves, from mathematical models, and the OD and pH values obtained by the samples A, B, C, D and E are showed in Figure 12.

Table 13. Results from hydrogen production batch tests, for the experiment nº 2 – "Run 1".

Sample name	**Hydrogen yield±SD [Nml H_2 / gVS]**	**Volatile Fatty Acids**			
		Acetic acid [mg/l]	*Propionic acid [mg/l]*	*Butyric acid [mg/l]*	*Isovaleric acid [mg/l]*
A	8.3 ± 0.4	99.0	7.1	2.8	23.0
B	11.9 ± 0.4	81.5	9.3	2.4	14.2
C	11.1 ± 0.5	85.6	6.0	2.6	8.4
D	9.6 ± 1.1	64.8	8.6	2.7	1.4
E	12.5 ± 3.6	80.1	9.5	4.6	4.1

Table 14. Mathematical model parameters, obtained by the sampled from the experiment nº2 – "Run 1".

Sample name	**Parameters of the Gompertz equation**		
	B_0 [Nml H_2 / gVS]	λ [d]	R [(Nml H_2) / (gVS*d)]
A	8.3	1.6	4.0
B	12.3	4.5	6.2
C	11.3	4.1	5.0
D	9.9	4.3	5.9
E	11.9	1.1	8.3

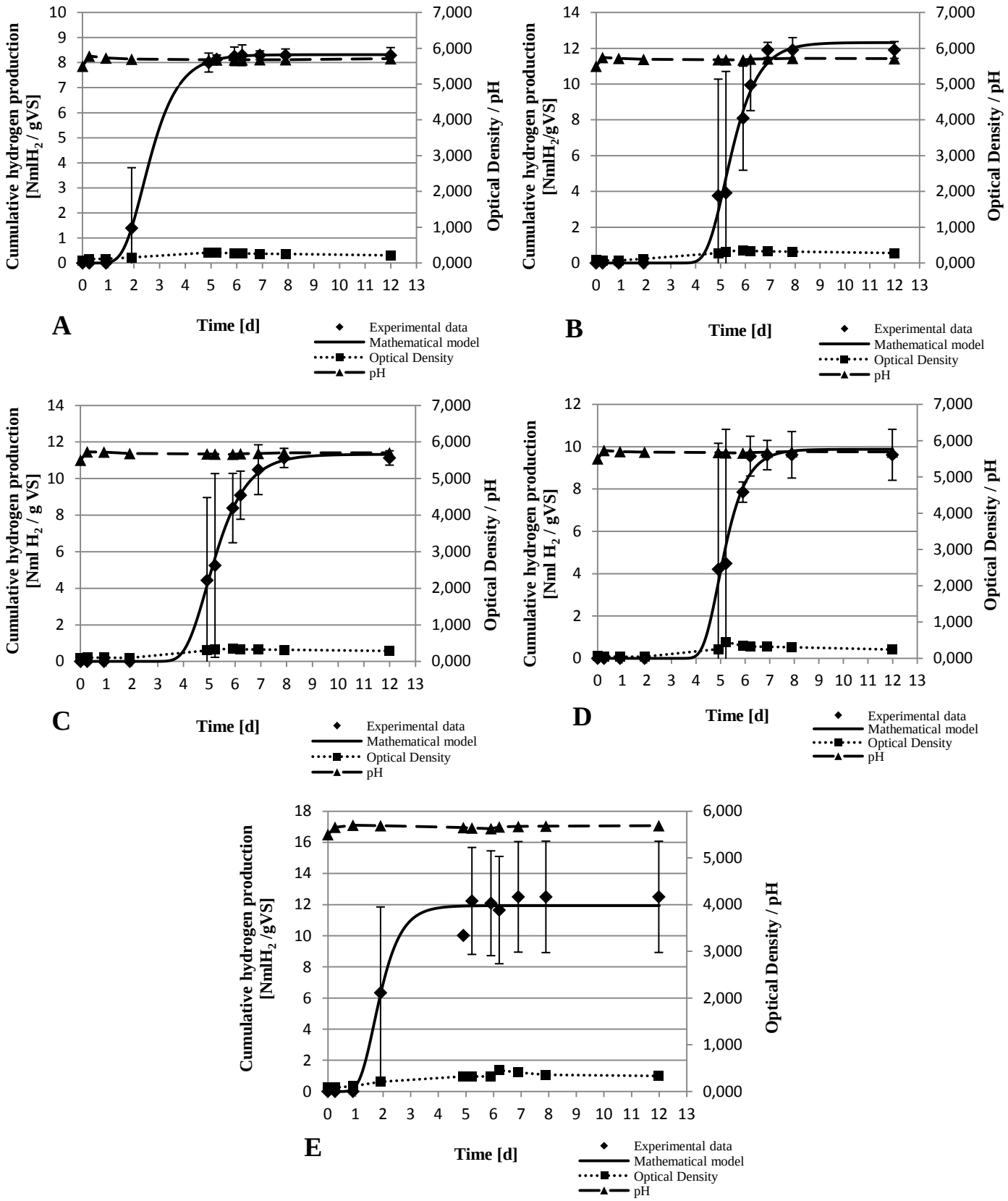

Figure 12. Optical Density and pH variations, over time, and cumulative hydrogen productions, in experiment nº2 "Run 1", from average experimental data and from the mathematical model by the samples: (A) *B. licheniformis*; (B) *P. cookie*; (C) *Bacillus* sp; (D) *Paenibacillus* sp.; (E) *Bacillus farraginis*. The vertical bars over the experimental data represent the standard deviations of the triplicate.

The highest hydrogen production was measured for *B. farraginis* (sample E), with a total hydrogen production of 12.5 NmlH_2/gVS, whereas *B. licheniformis* (sample A) showed the lowest production, with a total hydrogen production of 8.3 NmlH_2/gVS.

B. licheniformis and *B. farraginis* had a shorter lag phase than the other species, but although this difference the yields of hydrogen were very similar to each other (see Table 14).

An overall evaluation of all the samples, these results are much lower compared with those obtained in experiment nº 1 – "Run 1".

3.1.3.4 Experiment nº 2: "Run 2"

In this experiment glucose was used as substrate, with a concentration of 5 g/l, in each sample. The results of hydrogen production potentials are reported in Table 15. Even more, the mathematical model parameters, obtained from the average cumulative hydrogen productions of the experimental data, are described in Table 16.

The cumulative hydrogen productions curves, from mathematical models, and the OD and pH values obtained by the samples A, B, C, D and E are showed in Figure 13.

Table 15. Results from hydrogen production batch tests, for the experiment nº 2 – "Run 2".

Sample name	**Hydrogen yield±SD [Nml H_2 / gVS]**	**Volatile Fatty Acids**			
		Acetic acid [mg/l]	*Propionic acid [mg/l]*	*Butyric acid [mg/l]*	*Isovaleric acid [mg/l]*
A	76.7 ± 19.4	133.4	74.9	5.2	19.8
B	63.7 ± 16.4	144.7	5.8	4.5	14.1
C	84.5 ± 34.5	172.8	6.0	5.2	27.9
D	89.6 ± 37.8	154.3	7.2	4.4	17.5
E	95.2 ± 28.5	190.7	8.6	6.1	26.8

Table 16. Mathematical model parameters, obtained by the sampled from the experiment nº2 – "Run 2".

Sample name	**Exponential function parameters**		
	P_0 [Nml H_2 / gVS]	k [d^{-1}]	Max rate [(Nml H_2 / gVS)*d^{-1}]
A	76.7	1.3	99.7
B	63.7	1.3	82.8
C	84.5	0.8	67.6
D	89.6	1.3	116.5
E	95.2	0.9	85.7

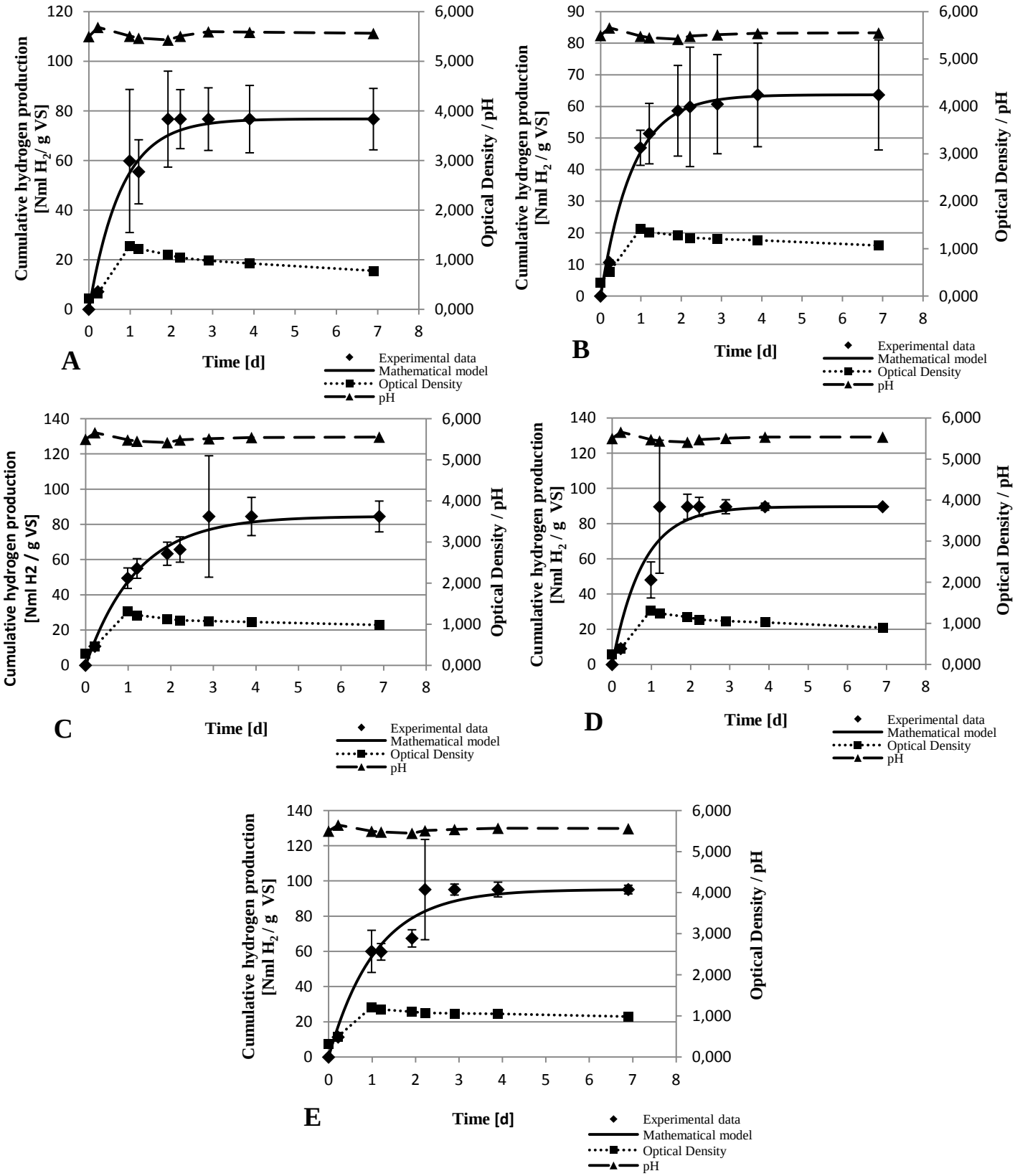

Figure 13. Optical Density and pH variations, over time, and cumulative hydrogen productions, in experiment nº2 "Run 2", from average experimental data and from the mathematical model by the samples: (A) *B. licheniformis*; (B) *P. cookie*; (C) *Bacillus* sp; (D) *Paenibacillus* sp.; (E) *Bacillus farraginis*. The vertical bars over the experimental data represent the standard deviations of the triplicate.

In this run the best results were obtained with the highest hydrogen productions. The maximum value was observed in the specie *B. farraginis* (sample E), with a total hydrogen production of 95.2 $NmlH_2/gVS$. In opposite, *B. licheniformis* (sample A) showed the lowest production, with a total hydrogen production of 63.7 $NmlH_2/gVS$. Even so, this value is higher than the values obtained in previous results.

All the samples showed similar behavior, which consists in a rapid increase in the hydrogen production, without lag phase, until reaching a maximum value of hydrogen production, from which the production practically ceased. At an early stage it is possible to observe a slight increase, which subsequently decrease again. Relatively to microbial growth, this shows a rapidly increase until achieved a maximum value.

3.2 Process performance

3.2.1 Overall evaluation of the results obtained by the pure cultures

In this section the results obtained in both experiments for the species *B. licheniformis* (sample A), *P. cookie* (sample B) and *Bacillus* sp. (sample C) will be compared.

In Figure 14 it is possible to simultaneously compare the behavior, that characterizes the hydrogen production process, obtained by the three samples in the experiment nº 1 – "Run 1" and bellow Figure 15 shows the results obtained from all the samples analyzed in the experiment nº 2 – "Run 1".

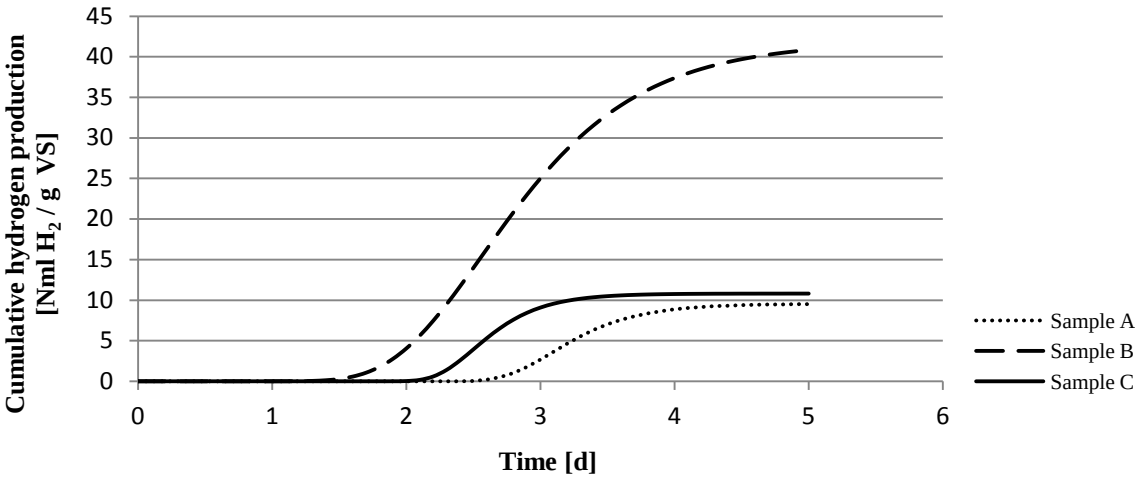

Figure 14. Comparison between the cumulative hydrogen productions from the mathematical model, obtained by the samples A, B and C in the experiment nº 1 – "Run 1".

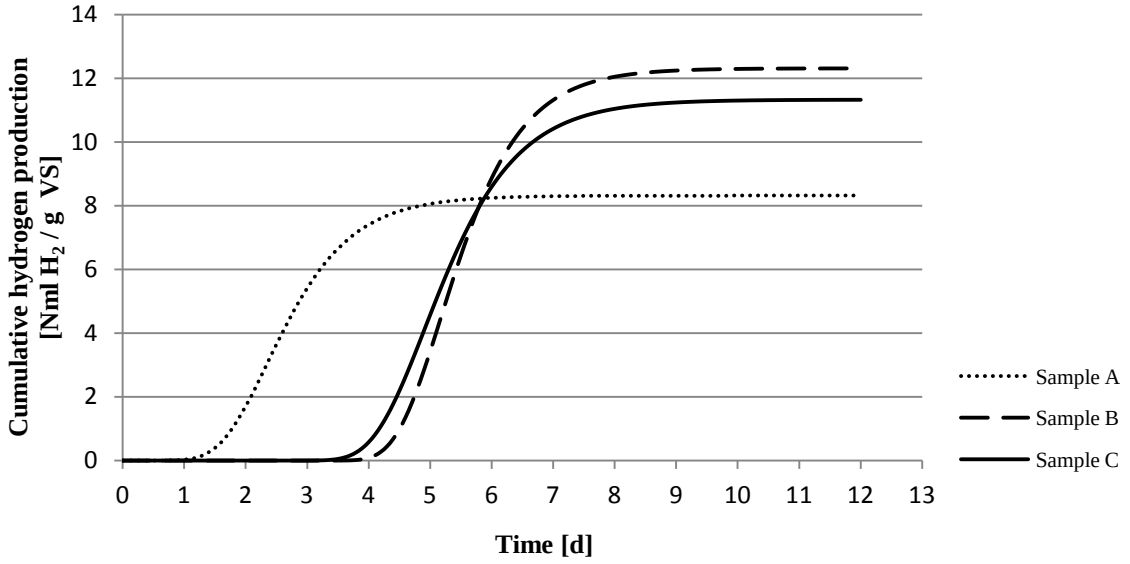

Figure 15. Comparison between the cumulative hydrogen productions from the mathematical model, obtained by the samples A, B and C in the experiment nº 2 – "Run 1".

From both figures a similar behavior can be observed consisting into three main phases: a lag phase, an exponential phase and a final phase.

During the first period the microorganisms were active but not yet favorable conditions were established to have the hydrogen evolution. This means that during the lag phase, the substrate used is mainly consumed for biomass growth. Thus, it is possible to suppose that during the lag phase electrons mainly flow towards biosynthesis and are not used for hydrogen evolution. The reason of this behavior remains to be explained. The second phase is characterized by exponential gas production, during which the medium composition changes but does not affect hydrogen production. Finally, during the third phase, hydrogen production stops due to the low quantity of substrate that remains in the reactor. (Ruggeri *et al*., 2009).

In another perspective, the hydrogen productions obtained in "Run 1", of both experiments, are represented in the Figure 16. In this situation, as previously mentioned, the difference lies in the substrate used – glucose (5 g/l) was added as substrate in the first experiment and NB (5 g/l) was added as substrate in the second experiment.

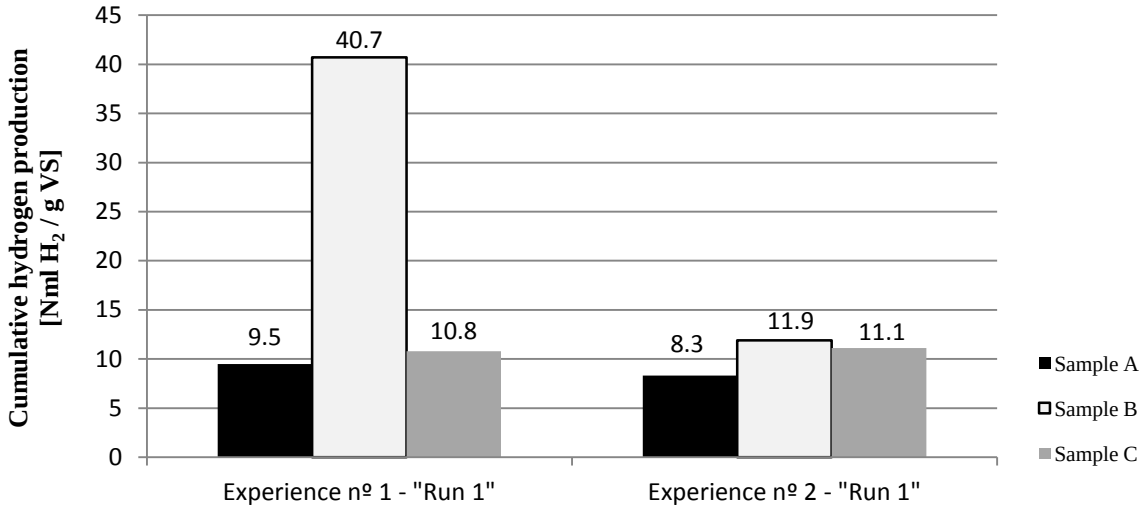

Figure 16. Hydrogen yields, from "Runs 1", obtained by the experiment nº 1 and by the experiment nº 2.

The results demonstrate that in the experiment nº 1 – "Run 1" occurred a higher hydrogen production by the specie *P. cookie*, named as sample B. This means that in this initial phase ("Run 1") there is a preference for the glucose, because it is an easily biodegradable carbon source. In opposite, bacteria showed difficulty in degrade the NB, once it is a complex substrate, for this initial state.

Despite the good result of the specie *P. cookie*, the other two species (*B. licheniformis* and *Bacillus* sp.) did not show a significant hydrogen production. The main reason for obtaining the yields lower than theoretical estimations is the utilization of the substrate as an energy source for bacterial growth (Kapdan & Kargi, 2006).

Figure 17 displays the yields of hydrogen obtained in "Run 2" of both experiments. In this case, the difference lies in the concentration of the substrate used. In experiment nº 1, the substrate used was glucose added with different concentrations, which were 5 g/l and 10 g/l. On the other hand, in experiment nº 2, only 5 g/l of glucose was added.

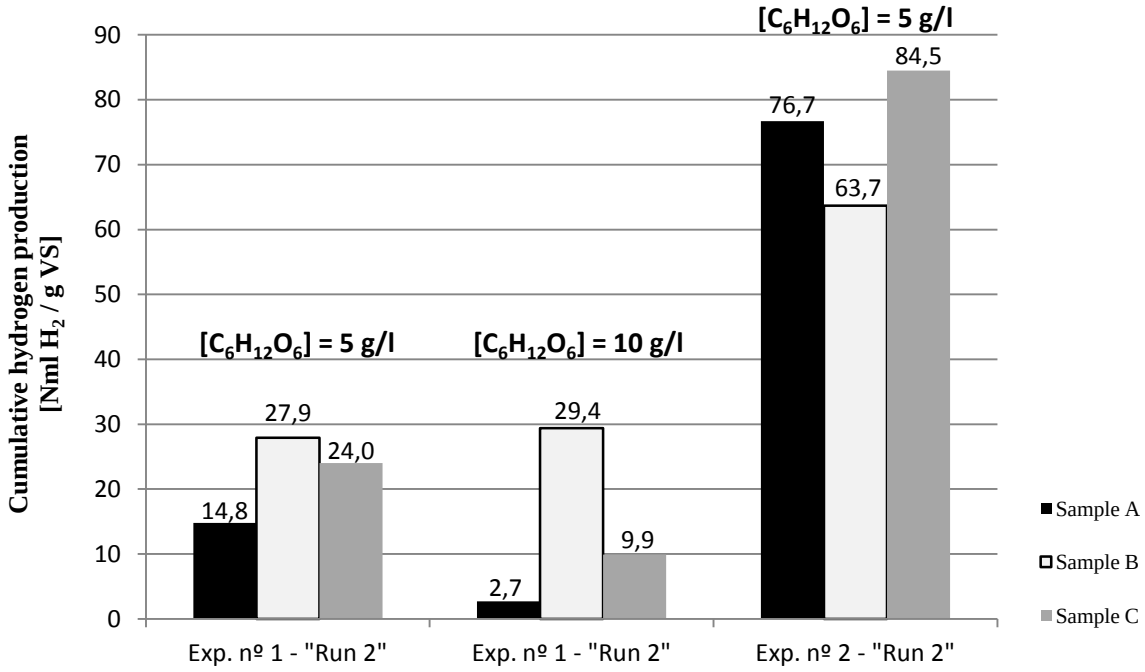

Figure 17. Hydrogen yields, from "Runs 2", obtained by the experiment nº 1 and by the experiment nº 2.

The results of experiment nº1 and nº 2, with glucose concentration set to 5 g/l, show higher hydrogen yields comparing to the experiment in which was used 10 g/l of glucose, this means that the efficiency of the hydrogen production was decreased by increasing glucose concentration, therefore high glucose concentration could inhibit the fermentation process. Many studies have reported similar results regarding the relationship of initial substrate concentration and hydrogen production (Hu *et al*., 2013).

It is important to consider that a proper ratio between C/N and C/P is essential for fermentative hydrogen production. In this sense, it is possible to conclude that the imposed substrate with the higher amount of carbon (10 g/l of glucose) supplied to the bacteria it is not necessary for their metabolism in these proportions, because it is inhibiting the biomass and consequently the H_2 generation in the reactors and so it is possible to minimize resources and achieve good results.

On the other hand, it was proven that the most efficient condition to obtain the higher hydrogen production is the addition of NB as substrate, in a first run, in order to provide the necessary nutrients for the bacteria, and subsequently add glucose as carbon source.

So, NB is providing all the nutrients that bacteria need to degrade the 5 g/l of glucose. In other words, it represents a good balance between the nutrients available and the substrate added. Figure 11 shows precisely these conclusions.

Therefore, can be pose pertinent questions for further researches: "How much hydrogen could be produced if a *Run 3* would be made?"; "This *Run 3* will show a faster rate of hydrogen production?".

It seemed that the highest hydrogen yields found in this study (Experiment nº 2 – "Run 2", see Table 15) were varied, but quite comparable to the yields of pure cultures in other studies. For comparison, Table 17 lists hydrogen yields obtained in this work and also from other studies that used glucose as substrate by pure cultures.

As is shown in Table 17, *Clostridium* and *Enterobacter* were most widely used as inoculum for fermentative hydrogen production. Species of genus *Clostridium* are gram-positive, rodshaped, strict anaerobes and endospore formers, whereas *Enterobacter* are gram-negative, rod-shaped, and facultative anaerobes (Guo *et al*., 2010).

As previously mentioned a lot of pure cultures of bacteria have been used to produce hydrogen from various substrates. Most of the studies using pure cultures of bacteria for fermentative hydrogen production were conducted in batch mode and used glucose as substrate; however, it is more desirable to produce hydrogen from organic wastes using pure cultures in continuous mode, because continuous fermentative hydrogen production from organic wastes is more feasible for industrialization to realize the goal of waste reduction and energy production. Thus more researches using pure cultures for continuous fermentative hydrogen production from organic wastes are recommended (Wang & Wan, 2009).

Table 17. The pure bacterial cultures for fermentative hydrogen production. [Adapted from: Hu *et al*., 2013 and Wang & Wan, 2009].

Inoculum	Substrate	Reactor type	Maximum hydrogen yield [mol H_2/mol glucose]	References
***Bacillus* sp.**	Glucose	Batch	0.6	This study
***Bacillus farraginis* LF 2.7**	Glucose	Batch	0.7	This study
***Bacillus licheniformis* LF 1.33**	Glucose	Batch	0.6	This study
***Citrobacter* sp. Y 19**	Glucose	Batch	2.49	(Evvyernie *et al*., 2001)
***Citrobacter amalonaticus* Y19**	Glucose	Batch	8.7	(Oh *et al*., 2008)
Clostridium acetobutylicum	Glucose	Batch	1.8	(Lin *et al*., 2007)
***Clostridium beijerinckii* L9**	Glucose	Batch	2.8	(Lin *et al*., 2007)
***Clostridium beijerinckii* DSM 1820**	Glucose	Batch	1.5	(Masset *et al*., 2012)
***Clostridium beijerinckii* RZF – 1108**	Glucose	Batch	2.0	(Zhao *et al*., 2011)
***Clostridium beijerinckii* DSM 791**	Glucose	Batch	0.6 – 1.6	(Hu *et al*., 2013)
***Clostridium butyricum* CWBI 1009**	Glucose	Batch	1.7	(Masset *et al*., 2010)
***Clostridium butyricum* ATCC 19398**	Glucose	Batch	2.3	(Kataoka *et al*., 1997)
***Colstridium butyricum* DSM 10702**	Glucose	Batch	2.4 – 3.1	(Hu *et al*., 2013)
***Clostridium paraputrificum* M – 21**	Glucose	Batch	1.1	(Jo *et al*., 2008)
Clostridium pasteurianum	Glucose	Batch	1.5	(Ferchichi *et al*., 2005)
***Clostridium pasteurianum* DSM 525**	Glucose	Batch	1.8 – 3.0	(Hu *et al*., 2013)
***Clostridium saccharoperbutylacetonicum* ATCC 27021**	Glucose	Batch	1.37	(Oh *et al*., 2003)
***Enterobacter aerogenes* HO – 39**	Glucose	Batch	1.0	(Yokoi *et al*., 1995)
***Enterobacter aerogenes* HU – 101 wt**	Glucose	Batch	0.6	(Mahyudin *et al*., 1997)
***Enterobacter aerogenes* DSM 30053**	Glucose	Batch	0.1 – 0.3	(Hu *et al*., 2013)
***Enterobacter cloacae* IIT – BT 08**	Glucose	Batch	2.2	(Kumar & Das, 2000)
***Escherichia coli* MC13 – 4**	Glucose	Batch	1.2	(Ishikawa *et al*., 2006)
Escherichia coli	Glucose	Batch	2.0	(Bisaillon *et al*., 2006)
***Paenibacillus* sp.**	Glucose	Batch	0.7	This study
***Paenibacillus cookie* LF 2.3**	Glucose	Batch	0.5	This study
Ruminococcus albus	Glucose	Batch	2.52	(Ntaikou *et al*., 2008)
***Thermoanaerobacterium thermosaccharolyticum* KU001**	Glucose	Batch	2.4	(Ueno *et al*., 2001)

3.2.2 Evaluation of the potential hydrogen productions from the mixed and the pure bacteria culture

The bacteria capable of producing hydrogen widely exist in natural environments such as soil, wastewater sludge or compost. Thus these materials can be used as inoculum for fermentative hydrogen production. At present, the mixed cultures of bacteria from anaerobic sludge, municipal sewage sludge, compost and soil have been widely used as inoculum for fermentative hydrogen production (Wang & Wan, 2009).

Fermentative hydrogen production processes using mixed cultures are more practical than those using pure cultures, because the former are simpler to operate and easier to control, and may have a broader source of feedstock. However, in a fermentative hydrogen production process using mixed cultures, the hydrogen produced by hydrogen producing bacteria may be consumed by hydrogen consuming bacteria. In addition, when mixed cultures are treated under harsh conditions, hydrogen-producing bacteria would have a better chance than some hydrogen-consuming bacteria to survive. Thus, in order to harness hydrogen from a fermentative hydrogen production process, the mixed cultures can be pretreated by certain methods to suppress as much hydrogen-consuming bacterial activity as possible while still preserving the activity of the hydrogen-producing bacteria (Wang & Wan, 2009).

Figure 18 shows the yields of hydrogen production obtained by the samples from the mixed and pure cultures, which achieved the best performance.

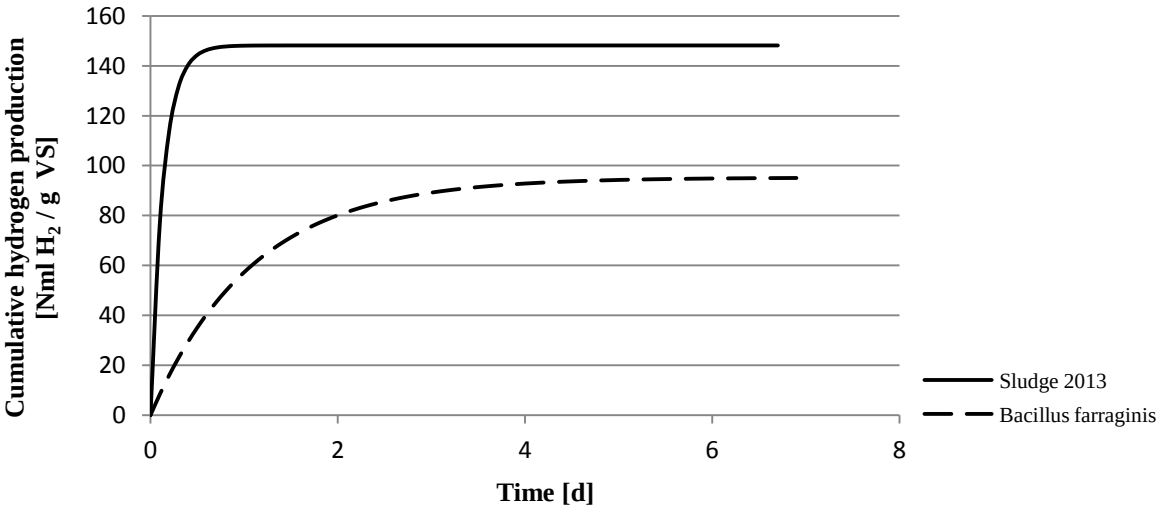

Figure 18. Cumulative hydrogen productions, from the mathematical model, obtained by the mixed and pure bacteria cultures, which are "Sludge 2013" and *Bacillus* sp., respectively.

The highest hydrogen production was measured by the mixed bacteria culture, represented by the sample "Sludge 2013", with a total hydrogen production of 148.2 $NmlH_2/gVS$. On the other hand, the pure bacteria culture, represented by *Bacillus farraginis* showed a total hydrogen production of 95.2 $NmlH_2/gVS$.

This means that the inoculum composed by the mixed bacteria culture induced to a higher H_2 production potentials. In fact, this result is expected since mixed culture is composed by different bacterial species, with vastly different taxonomic and physiological characteristics, which are cooperating each other allowing better results.

Nevertheless, pure culture had a good performance, since obtained a hydrogen production only less 36% than the mixed culture. This is actually a good result, because pure culture is composed only by one bacteria specie and so they are acting alone for hydrogen production.

There are some factors to be taken into account to achieve good results in both situations. The inhibition of hydrogen consumers present in the mixed cultures is essential for hydrogen production and for further scale-up and industrial application (Lee *et al.*, 2011). In turn, the pure cultures can be easily contaminated by other competitive bacteria and so the maximum sterilized conditions possible are required.

Therefore, the main purpose is to combine one pure culture with one real mixed culture to obtain an inoculum that is a consortium of bacteria, which is characterized by the higher potential for hydrogen production.

The vast majority of the hydrogen-producing microbial diversity however, is yet to be discovered. This unexplored biodiversity will be tapped as more research work is engaged in future and with setting up of mechanisms for integrated management and utilization of these microbial resources. The potential and strategies for harnessing microbial resources and their gene resources in dark fermentation could shed light in further improving the yield and production rates of hydrogen fermentations (Lee *et al.*, 2011).

4. CONCLUSIONS

The worldwide energy need has been increasing exponentially, the reserves of fossil fuels have been decreasing, and the combustion of fossil fuels has serious negative effects on environment because of CO_2 emission. For these reasons, many researchers have been working on the exploration of new sustainable energy sources that could substitute fossil fuels. In accordance with sustainable development and waste minimization issues, bio-hydrogen gas production from renewable sources, also known as "green technology" has received considerable attention in recent years. Therefore, production of this clean energy source and utilization of waste materials make biological hydrogen production a novel and promising approach to meet the increasing energy needs as a substitute for fossil fuels (Kapdan & Kargi, 2006).

The bacteria capable of producing biological hydrogen widely exist in natural environments and can be used as inoculum for fermentative hydrogen production. In this study, the mixed bacteria culture used as inoculum was from an anaerobic granular sludge. Furthermore, for the development of a specific inoculum five dark fermentation bacteria were investigated (*Bacillus licheniformis*, *Paenibacillus cookie*, *Bacillus* sp., *Paenibacillus* sp. and *Bacillus farraginis*), in order to compared their characteristics in hydrogen production.

The inoculum composed by the mixed bacteria culture, named as "Sludge 2013", induced to a higher H_2 production potentials. Nevertheless, the results that *Bacillus farraginis* showed were encouraging since obtained a hydrogen production only less 36% than the mixed culture.

Fermentative hydrogen production processes using mixed cultures are more practical than those using pure cultures, because the former are simpler to operate and easier to control, and may have a broader source of feedstock. However, in a fermentative hydrogen production process using mixed cultures, the hydrogen produced by hydrogen-producing bacteria may be consumed by hydrogen-consuming bacteria. The inhibition of these last microorganisms is therefore essential for achieving good results. Furthermore, sterilized conditions are required to obtain the best performance in the pure cultures.

Ensuring these ideal conditions, it is possible to obtain an inoculum that is a consortium of bacteria characterized by the higher potential for hydrogen production for further scale-up and industrial application.

In this study, the yields of hydrogen obtained by the pure bacteria, previously mentioned, were also compared using two types of substrates (glucose and NB) and changing the initial concentration in glucose.

The results showed that the efficiency of the hydrogen production was decreased by increasing glucose concentration, therefore high glucose concentration could inhibit the fermentation process. Moreover, it was proven that the most efficient condition to obtain the higher hydrogen production is the addition of NB as substrate, in a first run, in order to provide the necessary nutrients for the bacteria, and subsequently add glucose as carbon source.

The objectives initially proposed for this study were achieved. Thus, further research is necessary to better understand the impact of the composition of the substrate on biological hydrogen performances.

Therefore, future investigations may be interesting, taking into account the following pertinent questions: I) "*How much hydrogen could be produced using pure cultures if a further run would be made adding new substrate?*", II) "*Will this further run show a faster rate of hydrogen production compared to previous?*" and III) "*What is the best combination of pure culture species to achieve the highest hydrogen yields?*".

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ANNEX 1

Results of the BMP tests

Initially, batch tests were conducted for the evaluation of the sequential production of hydrogen and methane from the selected three specific types of granular sludge. The second phase of methane production was performed as soon as hydrogen production lasted.

For the BMP tests preparation, 50 g of granular sludge from 2013 were added in all the batch reactors available. Even so, the samples remained with the same identification: “Sludge 2011”, “Sludge 2012” and “Sludge 2013”.

The sludge from 2013 was selected because it showed, in the first part of this work, the best performance in terms of hydrogen production rate. It is important to note that the granular sludge used was not pre-treated prior to starting the experimental tests as inoculum of batch test for CH_4 production.

Moreover, to provide optimal conditions for methanogenic bacteria, the pH of the digestion liquid was raised from 5.5 to 7.5 by adding Na_2CO_3.

Anaerobic conditions were obtained by making nitrogen flow trough the head space of the vessel for 3 minutes. After this operation the excess pressure was removed in order to re-establish the atmospheric pressure. The mesophilic conditions were guaranteed by keeping the reactors in a water bath at a steady temperature of 35° C (± 1° C).

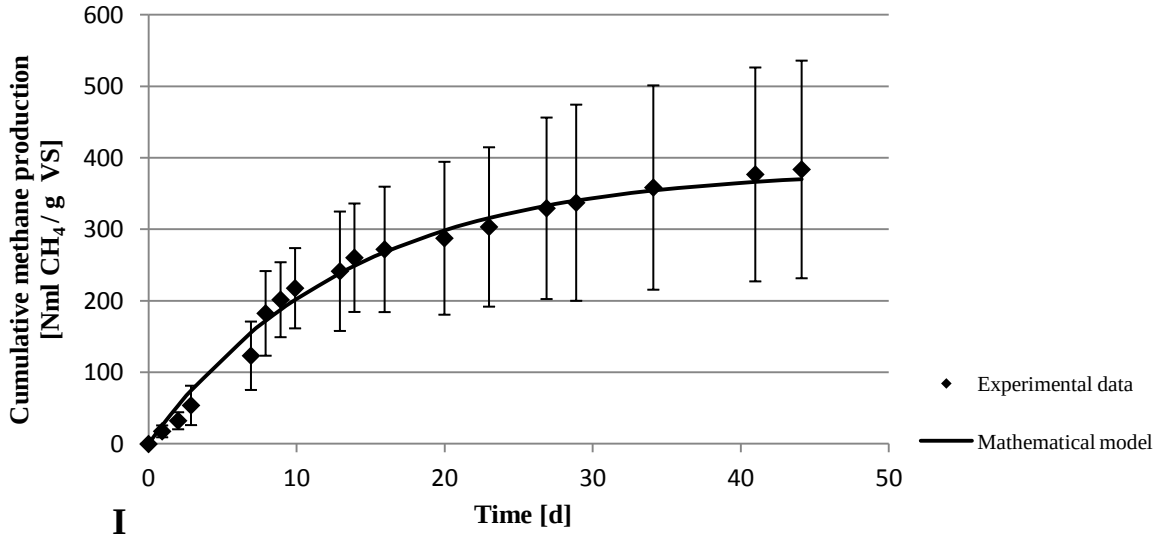
The amount of biogas produced was recorded daily, using the water displacement method and biogas composition in terms of hydrogen, carbon dioxide and methane was measured by a gas chromatograph, in this order quantity and the quality of the biogas were measured. The BMP tests took place over 45 days, after which no longer significant production of methane was noted.

Since an opportunity to establish an internal co-operation with colleagues from the Department of Agronomy Food Natural Resources Animals and Environment of the University of Padova came up, the hydrogen production became the main theme and so at the end of the BMP tests the experimental data were treated, in order to understand the behavior that each sample had at the second stage of the anaerobic digestion process. These results were used for comparison with other BMP tests results, obtained by a student who was doing her master's thesis in the laboratory. Furthermore these results can also be useful for future investigations in this field.

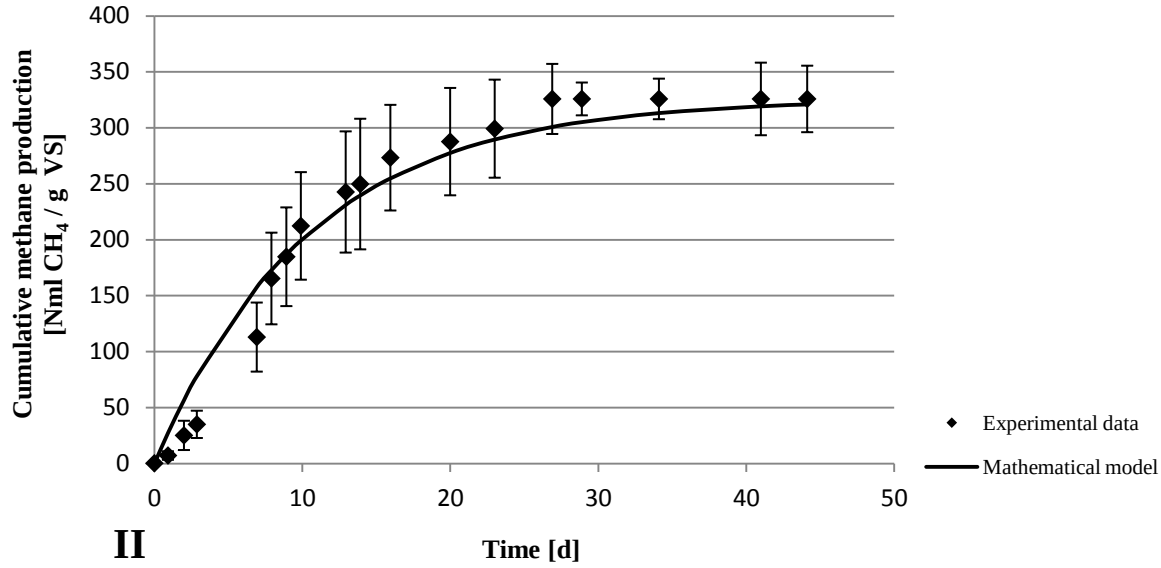
The results from methane production batch tests obtained by each sample and the mathematical model parameters, obtained from the average cumulative methane productions of the experimental data, are described in the following table.

Sample name	**Methane yield±SD [Nml CH_4 / gVS]**	**Exponential function parameters**		
		P_0 [Nml CH_2 / gVS]	k [d^{-1}]	Max rate [(Nml H_2 / gVS)*d^{-1}]
Sludge 2011	384.1 ± 152.2	384.1	0.08	30.7
Sludge 2012	325.6 ± 31.3	325.6	0.10	32.6
Sludge 2013	407.8 ± 41.8	407.8	0.04	16.3

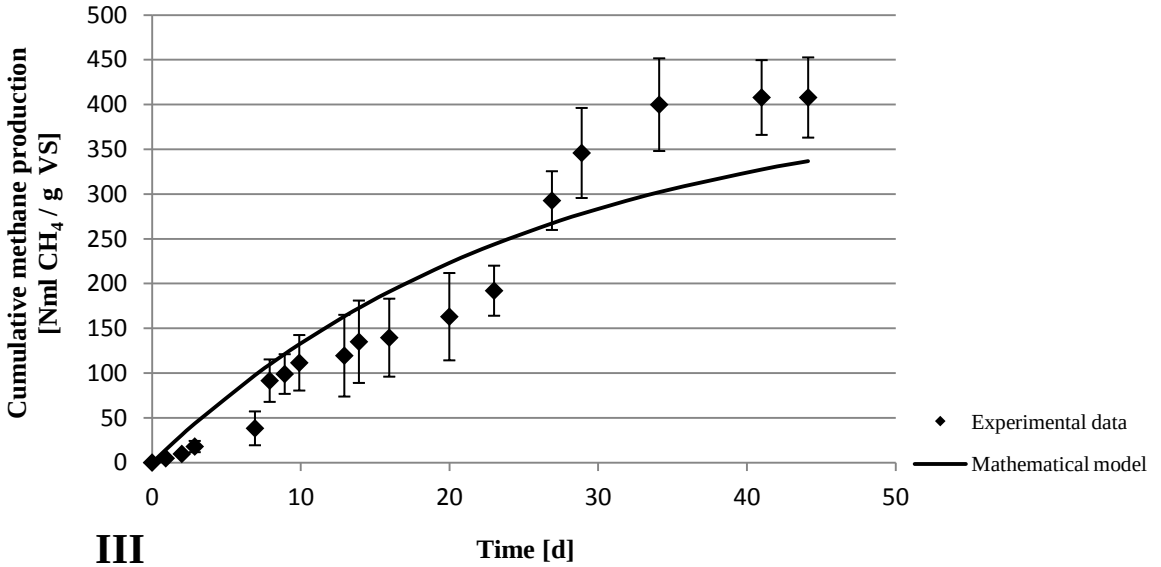
In the graphs above are represented the cumulative methane productions curves from experimental data average and from the mathematical model obtained by the three samples: (I) "Sludge 2011"; (II) "Sludge 2012"; (III) "Sludge 2013". The vertical bars over the experimental data represent the standard deviations of the triplicate.


Cumulative methane production [Nml CH_4 / g VS]
600
500
400
300
200
100
0
0
10
20
30
40
50
Time [d]
Experimental data
Mathematical model

I


Cumulative methane production [Nml CH_4 / g VS]
400
350
300
250
200
150
100
50
0
0
10
20
30
40
50
Time [d]
Experimental data
Mathematical model

II


Cumulative methane production [Nml CH_4 / g VS]
500
450
400
350
300
250
200
150
100
50
0
0
10
20
30
40
50
Time [d]
Experimental data
Mathematical model

III